STEROID HORMONE REGULATION OF EMBRYO DEVELOPMENT AND EMBRYO-MATERNAL COMMUNICATION FOR IMPLANTATION

By

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Dedicated to Andrés Alejandro, Kristina Elena, Milene, my mother, father and brothers.

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Abstract of Dissertation Presented to the Graduate School
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STEROID HORMONE REGULATION OF EMBRYO DEVELOPMENT AND EMBRYO-MATERNAL COMMUNICATION FOR IMPLANTATION

By

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Chairperson: Dr. Rosalia C. M. Simmen
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Pregnancy in the pig is characterized by high embryo mortality. This occurs during the first two weeks of gestation coincident with rapid embryonic morphological changes and embryonic secretion of high levels of estrogen. Embryonic estrogens act on the maternal uterus to stimulate synthesis and secretion of proteins required for successful pregnancy. However, the autocrine effects of estrogens on the embryo and the paracrine effects of androgens derived from maternal circulation or embryo on the uterus have not been reported. The objectives of this dissertation were to: 1) clone and characterize the porcine estrogen receptor β (ERβ) from developing embryos; 2) utilize porcine trophoblastic cell lines to evaluate estrogen effects on embryo growth; and 3) identify, characterize, and examine the function of androgen receptors in the early

pregnancy endometrium.

Porcine embryonic ERβ was cloned and its expression characterized during embryo development. Highest ERβ gene expression occurred at day 12 of pregnancy in filamentous embryos. Treatment of day 12 filamentous embryos with 17β estradiol (E) decreased expression of a gene (PCNA) correlated with growth and increased gene expression for ERβ and P450 aromatase. Porcine trophectoderm cell lines derived from days 12 and 14 embryos which express the ERβ gene showed decreased DNA synthesis and increased transcriptional activity of E-responsive reporter constructs, upon E-treatment.

The expression of the androgen receptor gene was identified in the endometrium throughout gestation. Maximal expression occurred between days 10 and 12 of pregnancy. Estradiol treatment of endometrial explants from day 10 pregnant pigs induced AR, IGF-I and PCNA gene expression whereas E, in combination with androgens, inhibited IGF-I and PCNA gene expression. Endometrial explants from day 12 pregnant pigs responded differently to steroid treatments, with an increase in IGF-I gene expression observed in the presence of E plus androgens. Primary cell cultures of glandular epithelial and stromal cells from early pregnancy endometrium had increased DNA synthesis with E and decreased DNA synthesis with testosterone. In conclusion, embryonic estrogens and maternal or embryonic-derived androgens act on the embryo and on the maternal endometrium, respectively to regulate gene expression and cell growth requisite for pregnancy success.

CHAPTER 1
INTRODUCTION

The pig is a polytococus species which produces an average of 10 -12 offspring per gestation. The gestation period lasts approximately 114 days. The first 2 weeks of pregnancy are characterized by dynamic changes in gene expression and corresponding protein secretion in the uterine environment, all of which are essential for the success of pregnancy (Roberts et al., 1993; Simmen et al., 1995). During this period a high incidence of embryo mortality occurs (Pope, 1985), and the causes of this remain unknown. Porcine embryos synthesize and secrete estrogens between days 11 and 12, which underlie the elevated levels of this steroid hormone found in the uterine lumen (Gadsby et al., 1980; Pusateri et al., 1990). These estrogens are known to function as the maternal recognition signal for pregnancy, and prevent luteolysis. Although embryonic estrogens are known to act on the uterus, they do not suppress this tissue ability to synthesize prostaglandin F2α ($PGF_{2\alpha}$), a luteolytic compound. Instead, embryonic estrogens change the direction of $PGF_{2\alpha}$ secretion from the vascular system to the uterine lumen (Bazer et al., 1986), preventing $PGF_{2\alpha}$ from causing corpora lutea to regress, and hence, pregnancy to continue.

One important feature of the peri-implantation period (days 11 and 12) is

rapid embryonic growth, occurring coincident with maximal embryonic estrogen production. It is fascinating to observe how a spherical embryo of 10 mm in diameter can change first into a tubular and then filamentous form, 100 mm in length, in less than 4 hours (Geisert et al., 1982). This extremely rapid growth process is still not well understood, and it is unclear which molecules present in the uterine lumen plays a major influence in this event. Numerous growth factors and steroids have been identified in the uterine luminal fluids (Stone and Seamark, 1985; Roberts et al., 1993; Simmen et al., 1995), and they have been postulated to affect embryonic growth. Indeed, peri-implantation embryos are known to express receptors for several growth factors including IGF-I, EGF, and KGF (Zhang et al., 1992; Green et al., 1995, Ka et al., 2001), however, the presence of steroid receptors in the developing embryo has yet to be reported. Since steroid hormone concentrations are very high during the time of embryo elongation, it is likely that they exert autocrine effects on developing embryos. Hence, the first objective of this dissertation is to demonstrate steroid receptor expression by developing peri-implantation embryos. Furthermore, the functional consequences of these receptors, if present, in the regulation of embryonic growth and development will be explored. Understanding the process of embryonic development may lead to important insight on the causes and mechanism underlying high embryo mortality in pigs.

The first logical step is to identify the presence of estrogen receptors (ER) in the embryo, due to the high levels of estrogen present within the uterine lumen. Several groups have tried to identify ER in porcine embryos during the

time of embryo elongation, but the results have been conflicting (Yelich et al., 1997; Dekaney et al., 1998). A role for embryonic ER in embryo mortality stems from the findings that estrogens administered into the uterine lumen before day 11 of gestation caused embryonic death (Morgan et al., 1987). However, estrogens given at the time of maternal recognition of pregnancy (between days 11 and 12) did not elicit the same effect. Moreover, the Chinese breed of pigs (Meishan) is known to produce more piglets per litter than the European Large White breed (Ford, 1997). A major difference between the two breeds is that Meishan embryos produce less estrogen than those of Large White; moreover, Meishan embryos grow slower and more synchronously than their Large White embryos (Wilson and Ford, 1997). These findings are consistent with estrogen acting as a likely factor in regulating embryo mortality.

In this dissertation, a newly characterized cell line generated from dispersed day 12 filamentous embryo trophoblastic cells will be used as an in vitro model to further characterize the biological effects of estrogen on embryo trophoblast. These cells provided by Dr. Laurie Jeager (Texas A&M University), have been characterized as derived from trophectoderm, and are known to exhibit an increased growth rate upon exogenous growth factor addition (Ka et al., 2001). However, the steroid receptor profile in these cells is unknown. Therefore, another objective of this dissertation is to characterize the expression of steroid receptors by this cell line, and to test the functional consequences of these receptors when bound to estrogen agonist or antagonist.

Recently, it has been reported that embryos not only synthesize estrogens

but also androgens, specifically 19-nortestosterone (Kao et al., 2000). This androgen likely comprises one of the long list of androgens present in the uterine lumen (Stone and Seamark, 1985). Androgens present in the uterine luminal fluid have long been considered to simply serve as substrates for embryonic P450 aromatase for estrogen synthesis, and are not likely to have any direct action on uterine function. Primates and rodents are known to exhibit the presence of androgens in their uterine luminal fluids, and to express uterine androgen receptors. Androgens have also been reported to regulate gene expression and growth of the uterus (Sahlin et al., 1994; Adesanya-Famuyima et al., 1999). Indeed, infertility problems have been related to elevated serum androgen concentrations in women and the presence of androgen receptors in their uteri (Okon et al., 1998). Therefore, the third objective of this dissertation is to identify and characterize androgen receptors in porcine uterine endometrium and its examine potential role in uterine gene expression and embryonic development.

CHAPTER 2
REVIEW OF THE LITERATURE

Steroid Hormone Receptors

Steroid hormone receptors belong to the nuclear receptor gene family of transcription factors which exhibit evolutionarily conserved structures (Laudet V., et al 1992). The nuclear receptor are classified into subfamilies such as the steroid receptor subgroup consisting of the receptor for glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens (Beato et al., 1996). The nuclear receptors comprise six subregions designated domains A through F. The N-terminal A/B region contains a hormone-independent transactivation function (AF-1) (Giguere et al., 1986; Kumar et al., 1987; Kato et al., 1995). The C region contains two zinc fingers, which are important for DNA binding and receptor dimerization (Freedman, 1992). The carboxyl terminus of the receptor is subdivided into a short region D called the hinge region and region E which is also called ligand binding domain (LBD) (Evans, 1988). The LBD has many functions such as association with heat shock protein, dimerization, nuclear localization, and hormone-dependent transactivation and/or repression (Tsai and O'Malley, 1994; Tsai and Tsai, 1997).

The actions of nuclear receptors involve the binding of the ligand to the receptor and the interaction of the ligand-receptor complex to cis-regulatory DNA elements in the promoter region of the target genes to stimulate transcription (Beato et al., 1996). The steroid receptors facilitate the formation of a stable pre-initiation complex through direct interactions of the steroid receptors with components of the basal transcriptional machinery, such as TATA binding protein and TFIIB (Tsai and O'Malley, 1994).

Members of the steroid hormone receptor superfamily function as dimers, usually homodimers that can be formed before or after hormone binding (Tsai et al., 1988). Amino acid sequences important to dimerization of the mouse estrogen receptor are located in a region of the ligand binding domain. Point mutations in this region reduced the ability of the receptor to form dimers and to bind DNA (Fawell et al., 1990). Monomeric, homodimeric, or heterodimeric modes of DNA binding result from receptor differences in the DNA binding and dimerization domains (Tsai et al., 1988a). The orphan receptor NGFIb is a good example of monomeric binding; this receptor binds with high affinity to response elements consisting of a single copy of a recognition sequence (Wilson et al., 1991). Steroid hormones receptors such as estrogen and glucocorticoid receptors bind as homodimers to respective response elements which consist of a pseudopalindromic arrangement of two recognition sequences (Kumar and Chambon, 1988; Tsai et al., 1988b). The DNA binding domain (DBD) from each of the dimer participants make contact with one of the recognition motifs resulting in a symmetric structure (Schwabe et al., 1993). Heterodimeric

interaction by nuclear receptors are demonstrated by retinoic acid receptor (RAR), thyroid hormone receptor (TR), and vitamin D receptor. These receptors heterodimerize with retinoid X receptor (RXR), and bind to response elements consisting of direct repeats, or palindromic or inverted palindromic arrangements, of recognition sequences (Yu et al., 1991; Kliewer et al., 1992).

Among the superfamily of nuclear hormone receptors, the DBD region is the most highly conserved domain (Freedman, 1992). Sequence analysis of several steroid receptors reveals a highly conserved cysteine-rich region within the DNA binding domain which folds into two zinc-finger structure (Evans and Hollenberg, 1988). The nuclear receptor DBD utilizes four cysteines in each of the two fingers to form two nonequivalent zinc fingers (Luisi et al., 1991).

Optimum binding of homo- or heterodimeric receptors to DNA requires the presence of two copies of a core recognition motif that are arranged to facilitate interactions between DBDs (Glass, 1994). The hormone response elements are generally composed of two repeats of core sequences arranged as palindrome, direct repeats, or sometimes as inverted repeats, showing that dimerization is important for DNA binding for most nuclear receptors (Freedman, 1992). In the glucocorticoid receptor, a symmetric interface is formed between the dimeric DBD bound to a palindromic glucocorticoid response element (Luisi et al., 1991). The distance between the DBDs allow the receptor dimers to produce an optimum protein-DNA contact (Dahlman-Wright et al., 1991).

Estrogen Receptors

Structure

The ERs are the first example of a steroid-hormone receptor existing as two isoforms where each isoform is encoded by a separate gene (Kuiper et al., 1996). Mammalian ER genes are located in different chromosomes; the human ERα gene is located on chromosome 6 (Menasce et al, 1993) and the mouse ERα gene is mapped to chromosome 10 (Sluyser et al., 1988). In contrast to that of ERα, ERβ

gene is localized on human chromosome 14 (Enmark et al., 1997), and on mouse chromosome 12 (Tremblay et al., 1997). Analysis of the human ERα gene has shown that it is a very large gene, with the open reading frame spanning nine exons and containing more than 140 kb (Ponglikitmongkol et al., 1988). The product of the human ERα is a protein of 595 amino acids with a molecular mass of approximately 66 kDa (Green et al., 1986). Transcription of the mouse ERα gene produce a single transcript of approximately 6.3 kb. The protein encoded by this transcript is 599 amino acids in length, and has a similar, albeit not identical molecular mass to the human ERα protein (White et al., 1987).

The exons of the human ERβ gene has been shown to span approximately 40 kb (Enmark et al., 1997). The full-length cDNA of human ERβ encodes 530 amino acids with a calculated relative molecular mass of 60 kDa (Ogawa et al., 1998). Several isoforms of ERβ gene have been characterized.

In rat, an ERβ containing a 54 nucleotide insertion in the LBD has been described and shown to have a reduced affinity for estrogens (Chu and Fuller, 1997; Petersen et al., 1998). In humans, several isoforms have also been described. Vladusic et al. (1998) reported a hERβ splice variant containing a 139 nucleotide deletion in the LBD, which is expressed in the estrogen-independent breast cancer cell line MDA-MB-231 and in malignant breast tumor specimens. Moore et al. (1998) characterized five different isoforms, and the predicted amino acid sequences of these five isoforms diverged at amino acid 469 within the LBD and extend to the C-terminus. These isoforms can form homodimers and heterodimers, although their tissue expression are quite distinct (Moore et al., 1998). The nucleotide boundaries are consistent with a 5' splice junction sequence, suggesting that the isoforms are generated by differential splicing of exons (Sharp, 1987; Moore et al., 1998).

Mechanism of Action

Estrogen receptors are classified as nuclear hormone receptor class I (Mangelsforf et al., 1995). Inactive ER exists in a complex with several heat shock proteins and immunophilin chaperones, which dissociate when the receptor is bound by the ligand (Pratt and Toft, 1997). The ERs are predominantly nuclear proteins, irrespective of whether they are bound to their ligand or not (Parker, 1995). When ERs are bound by ligands, a conformational change is induced leading to the formation of dimers (Wurtz et al., 1998). These ER-ligand dimers can interact with a response element, consisting of 15 bp

inverted palindromes in the vicinity of the target gene (White and Parker, 1998) and with basal transcription factors, coactivator proteins and other nuclear proteins to modulate rates of gene transcription (Tsai and O'Malley, 1994)

Ligand binding

Both ERα and ERβ have similarities in structure for the LBD (Saunders, 1998), and hence, exhibit similar responses to a number of ligands, although receptor-specific responses have also been noted (Barkhem et al., 1998). The overall homology between hERα and hERβ in their LBD is 59 % (Enmark et al., 1997). The ligand binding cavity of hERα is delineated by amino acid residues at positions 342 to 547. The homology between ERα and ERβ in their LBD is very high (~60%), specially with the amino acids within the cavity area (Brozowski et al., 1997). Therefore, 17β estradiol and other ligands bind to ERα and ERβ with similar affinities (Kuiper et al., 1997). Both types of ER are inactivated by the anti-estrogen ICI 182,780 and a novel nonsteroidal antiestrogen EM-800; however, the agonist effect of tamoxifen appears to be different depending on the ER subtype (Tremblay et al., 1997). Furthermore, the finding that ERα and ERβ respond differently to ligands through an AP-1 site suggest potential differences in control mechanism for genes regulated by estrogens, depending upon the ER isoform present (Paech et al., 1997).

Tamoxifen is one ligand which acts as a pure antagonist on ERβ but can be agonist or antagonist depending on the cell or tissue context for ERα (Watanabe et al., 1997; Tremblay et al., 1998). Also, it has been shown that

phytoestrogens such as coumestrol and the isoflavonoid genistein have up to ten times higher affinity for ERβ than for ERα (Kuiper et al., 1998). Another important aspect of the difference in response to antiestrogens is that in certain genes such as quinone reductase in colon cancer, the antiestrogen induces higher activity of this gene's promoter when bound to ERβ rather than ERα to mediate transcriptional response (Montano et al., 1998).

DNA binding

The most conserved region between ERα and ERβ is the DBD of the C-terminal domain, which is 97% homologous between human isoforms (Enmark et al., 1997). This region contains nine cysteine residues where eight of the residues are coordinated around two zinc ions that form two zinc finger motifs and allows the receptor to bind tightly to the DNA response element (Freedman, 1992). The sequence encoding the two zinc fingers in ERα and ERβ is located across exons 3 and 4 (Enmark et al., 1997; Tremblay et al., 1997). Residues within the first zinc finger are primarily responsible for determining target specificity (Green et al., 1988). However, both fingers, including the basic region immediately C-terminal to the second finger are important for DNA binding and nuclear localization (Picard and Yamamoto, 1987). Those residues within the C-terminal base of the first zinc finger are called the P-box which is formed by six amino acids including the third and fourth cysteines (Freedman, 1992). The DNA-binding specificity provided by the P-box was demonstrated by mutagenesis experiments. An estrogen receptor with three amino acid

substitutions in the P-box was transformed into a glucocorticoid receptor-like box, and was able to bind a glucocorticoid-responsive element instead of an estrogen-responsive element (Mader et al., 1989). Dimerization also enhances binding to DNA; this was demonstrated when the ERα DBD were dimerized, resulting in enhanced binding of the region to an imperfect estrogen-response element (Kuntz and Shapiro, 1997).

Dimerization

Steroid hormone receptors usually bind to inverted DNA repeats as homodimers, although the glucocorticoid and mineralocorticoid receptors have been shown to form heterodimers (Parker, 1993). Residues involved in the dimerization of the ERs are located in the second zinc finger of the C domain as well as in a region of the E domain called the major dimerization surface (Ylikomi et al., 1992). ERα and ERβ are capable of forming functional heterodimers on DNA both in vitro and in vivo (Cowley et al., 1997; Ogawa et al., 1998), and heterodimers bind to estrogen response element sequences with an affinity similar to that of ERα and greater than that of ERβ homodimers (Cowley et al., 1997).

When ERα and ERβ are expressed under conditions where heterodimers are the predominant dimeric species, transcription of an estrogen-response element reporter gene is stimulated to an intermediate level compared with that of either homodimer (Cowley et al., 1997). Petterson et al. (1997) showed that transcriptional activity of the mouse heterodimer when assayed in vitro is

between the more active ERα homodimer and the less active ERβ homodimer. Also, the transcriptional activity of a heterodimer is dependent on a functionally intact transactivational domain (AF-2) within both receptor subunits (Ogawa et al., 1998). It has been shown that both forms of receptor homodimers induce similar transactivation profiles in vitro when an estrogen-response element was used. In the same series of experiments, it was also observed that ERα and ERβ homodimer complexes with estradiol or several anti-estrogens signaled in opposite ways when they interact through an AP-1 site (Paech et al., 1997). Additional complexity in the activation of gene expression is that human ERβ splice variants can dimerize either with each other or with ERα before binding to an estrogen response element (Moore et al., 1998). However, these ERβ splice variants have lower affinity for 17β estradiol than the longest form of ERβ (Petersen et al., 1998)

Transactivation

Two distinct regions within the ER contribute to its transcriptional activity. These are the constitutively active amino-terminal activation function (AF-1) region and the ligand-regulated AF-2, which is located in the carboxyl-terminal part of the molecule (Smith, 1998). Depending on the cell type, target promoter, or the ligand type, AF-1 and AF-2 may regulate transcription independently or synergistically (Tzukerman et al., 1994). The AF-1 is a region of site-specific phosphorylation involved in ligand-independent activity of the receptor (Bunone et al., 1996). Activation of ER by estrogenic ligands is associated with the

increase in overall receptor phosphorylation (Arnold et al., 1994; Goff et al., 1994). The AF-2 is critical to the ligand-dependent transactivational activity of the receptor, where ligand-induced conformational changes create the functional AF-2 surface (Pettersson and Gustafsson, 2001). Even though the AF-1 and AF-2 can function independently, their functional interaction is required for full activity of ERα (Gandini et al., 1997).

Cowley and Parker (1999) isolated AF-1 and AF-2 regions from ERα and ERβ, and fused these to the DBD of GAL-4. AF-1 from ERβ was unable to initiate transcription by itself, in contrast to ERα AF-1 during cotransfection experiments. However, AF-2 from ERβ induced transcription from a TATA-containing promoter but less efficiently than AF-2 from ERα. When AF-2 from each receptor was cotransfected with a thymidine kinase promoter, they were equally potent in transcriptional activity. Thus, both receptors appear to contain a functionally conserved AF-2, and ERs have been shown to be stimulated by binding with coactivator SRC-1 in the presence of the ligand (Cowley et al., 1997; Tremblay et al., 1997). ERα and ERβ show opposite effects in regulation of AP-1 in an AF-1 dependent manner (Webb et al., 1999). Anti-estrogens induce activity of a promoter containing an AP-1 site in the presence of ERβ, whereas estrogen blocks transcription, and in the presence of ERα the pattern is reversed (Paech et al., 1997). All these findings suggest that the distinct transactivational mechanism operating between ERα and ERβ are due to their different AF-1 and AF-2 functions.

Coactivators, cointegrators and corepressors

Nuclear receptors coordinate diverse physiological roles in metabolism and development through ligand-dependent and -independent mechanisms (Glass and Rosenfeld, 2000). Coactivators are proteins that facilitate communication between nuclear receptors, the basal transcriptional machinery, and the chromatin environment (Torchia et al., 1998). Several factors increase ER-mediated transcription, and one group of factors is referred to as p160 or SRC (Steroid receptor coactivators) (Mckenna et al., 1999). The interaction of cofactors with nuclear receptors depends on the nuclear receptor-box within the cofactors. This box is composed of a consensus sequence LXXLL where L is a leucine and X is any other amino acid (Henttu et al., 1997). Three LXXLL-nuclear receptor box motifs are conserved in all members of the SRC family (McInerney et al., 1998). Hormone-dependent transcriptional activation of ER and other nuclear receptors involves assembly of a coactivation complex, which include the SRC proteins, CREB binding protein (CBP) and the related factor p300 (Mckenna et al., 1999). Assembly of SRC members and CBP/p300 with nuclear receptors in order to form a receptor coactivation complex, has been shown to occur in the presence of ligand (Robyr et al., 2000).

Several proteins have been reported to interact with ER AF-2 domain, some of which have the properties of enhancing transcription. One of these is a member of the SRC family, the SRC-1 (Onate et al., 1995). Recruitment of the SRC-1 protein to the ERs is dependent on the integrity of a C-terminal helix, referred to as helix 12, and a lysine residue in helix 3 of the ER (Feng et al.,

1998). Additional interactions between SRC proteins and AF-1 have also been reported (Webb et al., 1998). The SRC-1-ERα interaction has been mapped to a cluster of residues in the LBD of the receptor, which comprises an interaction surface that allows the docking of the LXXLL nuclear receptor box motif (Mak et al., 1999).

It has been reported that the increase in transcription by SRC proteins and ER can occur in the absence of the ligand, and comparison between both ERs has shown that ERβ is more responsive than ERα to cellular levels of SRC-1 in the absence of ligand (Tremblay and Giguere, 2001). Additional levels of complexity in ER mediated signaling have been shown with the discovery of cointegrators CBP and the related form p300 (Chakravarti et al., 1996). CBP/p300 assemble in a ligand-dependent manner with the estrogen-receptor-coactivator complex (Hanstein et al., 1996). CBP/p300 acts synergistically with ligand activated ERα in enhancing in vitro transcription of chromatin templates (Kraus and Kadonaga, 1998). CBP also potentiates the effects of SRC-1 on the transactivation properties of ERβ in the absence of ligand, while ERα was poorly affected under the same conditions. In the same set of experiments, the recruitment of SRC-1 to phosphorylated ERβ was enhanced by CBP (Tremblay and Giguere, 2001). These sets of experiments support the idea of the role of CBP in stabilizing ERβ AF-1/SRC-1 complex in the absence of ligand.

Histone acetylation activity has been shown to be present in these coregulator and cointegrator proteins (CBP/p300, SRC-1) (Kadonaga, 1998). These proteins can also acetylate transcription factors such as tumor suppressor

p53 (Liu et al., 1999), and the Kruppel-like factor I (Zhang and Bieker, 1998), and enhance their activities . In recent studies, it was demonstrated that ERα is acetylated in vivo by p300, resulting in the up-regulation of its hormone-dependent transactivation function (Wang et al., 2001).

Several factors repress transcriptional activity in nuclear receptors. Nuclear receptor corepressor (NcoR) and Silencing Mediator for Retinoid and Thyroid receptor (SMRT) have been shown to interact with and repress transcriptional activity of nuclear receptors (Chen and Evans, 1995). NCoR and SMRT are components of a complex that contains SIN3 and histone deacetylase (Alland et al., 1997). SIN3 is a protein that interacts with nuclear proteins, and serves as an intermediary between the receptor and histone deacetylase itself (Wong and Privalsky, 1998). NCoR interacts with the non-ligand bound receptor and upon ligand binding the corepressor is released and the interaction with coactivators can occur (Nagy et al., 1999). SMRT corepresses the activity of ER and PR but only in the presence of tamoxifen and RU486 respectively (Smith et al., 1997; Wagner et al., 1998). The ability of corepressor to interact only with ERα and PR in the presence of their antagonistic ligand suggests that steroid receptors contain a corepressor-binding site that is exposed in the antagonist-binding receptor conformation (Wagner et al., 1998). Another protein that acts as a repressor for ERα and ERβ is the orphan receptor SHP (Short Heterodimer Partner) (Johansson et al., 1999). The SHP interacts with ERs and antagonize binding of p160 coactivators to the ERs (Johansson et al., 2000).

Signaling by phosphorylation

Phosphorylation of ERs occurs as part of both the ligand-induced activity and the ligand-independent transcriptional activation (Denton et al., 1992; White et al., 1997). When ERs are activated by estrogenic ligands, this is associated with the increase in overall receptor phosphorylation (Ali et al., 1993). There are four sites of phosphorylation on the A/B domain, which are serine residues (LeGoff et al., 1994). A conserved tyrosine in the LBD in both ERs, which is located at position 537 in human ERα, is phosphorylated by a tyrosine kinase independent of estrogen ligands (Arnold et al., 1995). Studies using site-directed mutagenesis have shown that substitution of the Tyrosine 537 for a serine or alanine produces a constitutively active receptor (Weis et al., 1996). This mutant also showed an estrogen-independent interaction with SRC-1 which enhanced even more the ligand-independent transcriptional activity (Shiau et al., 1998). Mutation of the corresponding tyrosine to asparagine in ERβ LBD also resulted in a receptor that is active without ligand (Tremblay et al., 1998).

Phosphorylation of serine residues of human ERα are induced by epidermal growth factor (EGF) via MAPK and occurs in a ligand-independent manner (Bunone et al., 1996). However, estrogen can induce phosphorylation of serine 118 independent of MAPK (Joel et al., 1998). Serine 104 and 106 in human ERα are phosphorylated by the Cyclin A-cdk2 complex, enhancing transcriptional activity of ERα in both the absence and the presence of ligand (Rogatsky et al., 1999). ERβ transcriptional activity has been shown to increase in a dose-dependent manner with EGF, insulin like growth factor-I (IGF-I) and

transforming growth factor α (TGFα) treatments in the absence of ligand. In the presence of estrogen, increasing doses of EGF contributed to an additive activation of ERα and ERβ mediated transcription (Tremblay and Giguere, 2001). In addition, the phosphorylation of serine residues on ERβ by MAPK resulted in ligand-independent recruitment of SRC-1 and an increase in transcriptional activity (Tremblay et al., 1999).

Other compounds such as forkskolin, okadaic acid and cholera toxin can induce ER transcriptional activity in the absence of ligand by increasing the phosphorylation status of ER (Aronica and Katzenellenbogen, 1993). Increase cellular content of cAMP stimulates ligand-independent transcriptional activity of ERα and also synergize with estrogen-mediated activation (Ince et al. , 1994). Activation via cAMP signaling pathways requires AF-2 and is dependent on PKA (El-Tanani and Green, 1997).

Physiological Role of Estrogen in Reproduction

Female Reproductive Tract

ER is known to play a crucial role in several reproductive processes, including the control of reproduction, reproductive behavior and the development of secondary sexual characteristic in animals (Evans, 1988). The uterus has been shown to have a high affinity binding for radiolabeled estradiol as well as to have measurable physiological response upon exposure to the hormone (Murphy and Ghahary, 1990; Greco et al., 1993). Treatment of neonatal female

mice with estrogen showed stimulation of protein synthesis and cell proliferation in whole uterine tissues (Ogasawara et al., 1983). However, in mature uterus the response to estrogen is limited to the epithelial cells (Quarmby and Korach, 1984).

In the ERα knockout mouse, the myometrium and the endometrium of the uterus are underdeveloped and smaller than those of wild type mice (Lubahn et al., 1993). In the ERβ knockout mouse, the uterus appears normal and responds to the normal changes associated with the pattern of secreted hormones during the reproductive cycle (Krege et al., 1998). It has been shown that ERβ acts as a modulator of ERα-mediated gene transcription in the uterus, and furthermore, that it is responsible for down-regulation of PR in the luminal epithelium (Weihua et al., 2000). In the ER double knockouts (ERα and ERβ), the functional uterine compartments are present; however, they show a severe hypoplasia in adults (Couse et al., 1999). The response of ovariectomized wild type mice to estradiol are shown to be biphasic; in the first hours there is an increase in water imbibition, vascular permeability, and glucose metabolism, followed by a second phase of increases in RNA and DNA synthesis, mitosis, and cellular hyperplasia and hypertrophy (Couse and Korach, 1999). The ERα knockout mouse does not exhibit any of these responses suggesting that they are mediated mainly in the uterus through ERα (Couse et al., 1995). However, not all these changes in the uterus are a direct effect of estradiol-ERα complex; some of them are mediated by stimulation of growth factors such as EGF and IGF-I (DiAugustine et al., 1988; Murphy and Ghahary, 1990), and their respective

receptors. The knockout mouse for EGF receptor has similar characteristics to that of ERα knockout mouse in uterine development (Horn et al., 1998). The importance of a cross talk mechanism between EGF and ERα signaling pathway has been demonstrated since treatment with an ER inhibitor reduced the effect of EGF treatment (Ignar-Trowbridge et al., 1992) and blocking EGF action through its specific antibody reduced uterine response to estradiol (Nelson et al., 1991). The ERα knockout female mice are completely infertile (Korach et al., 1996). However, ERβ knockout female mice develop normally but have reduced fertility (Krege et al., 1998).

Other important targets for estrogen in the reproductive female tract are the ovaries, where estrogen induces a significant increase in ovarian weight in the hypophysectomized rat (Rao et al., 1978). In the granulosa cells of the growing follicle, estrogen increases its own receptor, and induces DNA synthesis as well as cell proliferation (Bley et al., 1997). Other important effects of estrogen in the follicle are the increase in IGF-I synthesis (Hernandez et al., 1989), and the decrease in apoptosis and follicular atresia (Kaipia and Hsueh, 1997). Estrogen acts to enhance responsiveness of the follicle to gonadotropins, which result in increased aromatase activity and estrogen production (Wang and Greenwald, 1993). ERβ mRNAs are predominantly localized in the granulosa cells of the growing follicle (Byers et al., 1997), while those of ERα are localized to the theca cells (Sar and Welsch, 1999). During the process of final growth of the follicle and ovulation in rat, ERβ gene expression is high until the LH surge occurs. The LH surge results in decreased levels of ERβ in the granulosa cells,

allowing progesterone receptors to increase and ovulation to occur (Byers et al., 1997). In the PR knockout mouse, the ovaries showed an inability of follicles to ovulate (Lydon et al., 1995). These results suggest the importance of the down-regulation of ERβ to allow the subsequent increase of PR expression and hence, ovulation to occur. Schomberg et al. (1999) described that in the ERα knockout mouse, the ovary of the neonatal and prepubertal female are normal. The mature females do not ovulate, and the phenotype of the ovary is enlarged, hemorrhagic, abundance of cystic follicles, and absence of corpora lutea. On the other hand, preantral and tertiary follicles are present, indicating that ERα is not necessary for the recruitment of primordial follicles.

The ERβ mRNA levels remain relatively constant during the follicular stage of the rat ovarian cycle but are rapidly decreased after gonadotropin surge which induces luteinization of the granulosa cells (Byers et al., 1997). The ERβ knockout female mice do not exhibit any abnormal phenotype during neonatal or mature stages of development. However, these mice produce fewer litters as well as less numbers of pups per litter (Krege et al., 1998). The reduction in fertility of the ERβ knockout is related to a reduction in completed folliculogenesis, since there is an increase in atretic follicles and a reduction in corpora lutea (Dierich et al., 1998; Krege et al., 1998). In the ERα-ERβ knockout female mice, the ovaries of adult females exhibit structures that resemble seminiferous tubules of the testis, Sertoli-like cells which express Mullerian inhibiting factor. These results suggest that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary (Couse et

al.,1999).

Other reproductive targets for estrogen are the vagina, cervix and oviduct (Kronenberg and Clark, 1985; Yamashita et al., 1989; Gorodeski, 1998). Estrogen targeting of the vagina promotes differentiation of the stromal cells and stimulates rapid proliferation of the epithelial cells, resulting in a cornified epithelial layer (Galand et al., 1971). Estrogen has also been shown to increase cervical secretions in women (Gorodeski et al., 1998) related to an increase in cell permeability (Gorodeski and Dipika, 2000). In the ERα knockout female mice, the vagina does not respond to estrogen (Korach et al., 1996). However, the vagina of the ERβ knockout mouse undergoes normal changes associated with the ovarian cycle (Krege et al., 1998). .

In the mouse oviduct, estrogen receptors are also present, and reach maximum levels by the proliferative stage (Yamashita et al., 1989). In rabbits, estrogen increases protein synthesis in the oviduct (Roy et al., 1972). In pig oviducts, maximum levels of protein synthesis are correlated with peak levels of estrogen during the estrous cycle (Buhi et al., 1996). However, no aberrant phenotypes were found in the oviduct of either ERα or ERβ knockout mice (Korach et al., 1996; Krege et al., 1998).

Male Reproductive Tract

Exposure to estrogens during the prenatal period results in a series of abnormalities within the male mouse reproductive tract such as undescended

testes, increased expression of estrogen-responsive genes, and sterility (McLachlan et al., 1975; Beckman et al., 1994). Also, excessive exposure to estrogens during development causes prostatic neoplasia (Bosland et al., 1995).

ERα has been localized to Leydig cells and Leydig cell precursors (Zhai et al., 1996). ERα was also immunolocalized to the rete testis, the epithelium of the efferent ductules, and within the epididymis (Fisher et al., 1997). ERβ mRNA and protein are expressed in Leydig cells, elongated spermatids, efferent ductules, and the initial segment of the epididymis in mouse (Rosenfeld et al., 1998). In the rat, ERβ mRNA is expressed in the prostate gland (Kuiper et al., 1996), and in the Sertoli cells within the seminiferous epithelium (Saunders et al., 1997). In the ERα knockout male mice, the reproductive tract undergoes normal prenatal development, however, four months after birth there is a significant decrease in the testis and epididymal weight (Eddy et al., 1996). The ERα knockout male mice are infertile due to the lack of normal sexual behavior, lower sperm counts, sperm with low motility and incomplete ability to fertilize oocytes in vitro (Eddy et al., 1996). These knockout mice showed significant atrophy of the seminiferous epithelium, and when they are 5 months old, the mouse testis is completely atrophied due to the continued increase in pressure within it that reduces its blood supply (Eddy et al., 1996). Histological analysis of the 60 and 90 day old ERβ knockout male mice showed no evident abnormalities, however, older males exhibited epithelial hyperplasia in the prostatic collecting ducts as well as the bladder walls (Krege et al., 1998). Studies have shown that ERβ knockout mice have elevated levels of androgen receptors compared to the wild

type. Also, treatment of wild type males with estrogen reduced the levels of androgen receptor, but this was not the case for the knockout, suggesting that ERβ regulates androgen receptor levels in and growth of the prostate gland (Weihua et al., 2001). After estrogenization of wild type male rats, these animals are more susceptible to estrogen-induced carcinogenesis of the urogenital tract, and this condition leads to increase expression of ERα, and decreased expression of ERβ in ventral prostates of adults animals (Prins et al., 1998).

Nongenomic Estrogen Signaling

In the classical action of estrogen, these steroids must enter the cell, bind their nuclear receptor to form a complex which can then bind DNA and activate gene transcription. These events occur within minutes to hours. However, there are estrogen-mediated effects that occur within miliseconds to minutes that cannot be explained by the classical model of estrogen action (Moss et al., 1997). These rapid, steroid-mediated effects are referred to as nongenomic signaling responses and are usually characterized by several criteria: 1) signaling responses occur within seconds to minutes; 2) responses are refractory to inhibitors of transcription (actinomycin D) and translation (cycloheximide); and 3) responses can be initiated by steroid conjugated to macromolecules [bovine serum albumin (BSA)] which are too large to enter cells (Coleman and Smith, 2001).

It has been shown that estrogen induces changes in cytoplasmic Ca^{+2}

within seconds, and these changes can be inhibited by removing Ca^{+2} from the extracellular medium or by treating cells with Ca^{+2} channel blockers (Han et al., 2000). These results indicate that Ca^{+2} influxes are mediated by membrane Ca^{+2} channels(Han et al., 2000). The same effect has now been demonstrated in many cell types including rat duodenal enterocyte cells (Picotto et al., 1999). Estrogen can also rapidly activate extracellular-regulated kinase (Erk1/Erk2) and can influence c-Jun kinase (Jnk) activity (Migliaccio et al., 1998). Razandi et al. (2000) showed that estrogen-BSA conjugated also induces Jnk cellular activity via nongenomic mechanisms. The inability of estrogen-BSA conjugated to enter cells was correlated with the ability of estrogen, but not of estrogen-BSA to stimulate ERE-dependent reporter activity (Razandi et al., 2000).

The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) dependent signaling pathway can also be rapidly stimulated by estrogen (Beyer and Karolczak, 2000). Treatment with either estrogen or conjugated estrogen-BSA promotes development of dopaminergic neurons, and these effect can be blocked by an inhibitor of PKA (H89) (Beyer and Karolczak, 2000). Endothelial nitric oxide synthase (eNOS) activity is rapidly stimulated by estrogen, and this activation was inhibited by the estrogen antagonist ICI 182,780 (Shaul, 1999). The ability of estrogen to stimulate eNOS was recently shown to be associated with ERα-mediated activation of the phosphatidylinositol-3-OH (PI-3) kinase-AKT signaling pathway (Simoncini et al., 2000). It has also been shown that proteins unrelated to the classical ER mediate binding of estrogen to the membrane and induces Ca^{+2} fluctuations in mouse macrophages (Benten et al., 2001). Using a

fluorescen-tagged estrogen (E-BSA-FITC), Benten et al. (2001) were able to detect membrane binding to this complex; however, neither ERα nor ERβ antibodies were able to bind to these membrane fractions, additionally, the E-BSA-FITC was internalized into the cytoplasm over a short period of time, suggesting that estrogen-binding protein can undergo internalization from the extracellular surface to the cytoplasm (Benten et al., 2001). Together, these reports suggest that the nongenomic effects of estrogen may function through the estrogen classical receptor or through a new estrogen-binding protein associated to the membrane.

Androgen Receptor

Structure

The Androgen receptor (AR) is a member of the superfamily of nuclear receptors. The human AR gene maps to the X chromosome in the q11-12 region and encodes a 110 Kda protein composed of 919 amino acids (Chang et al., 1988). This gene comprises more than 90 kb of DNA containing 8 exons, and the transcription of this gene produces a mRNA of about 11 kb (Brown et al., 1989). Another form of AR has been isolated with a molecular mass around 87 kDa. This new form was named AR-A and the previous form was named AR-B (Wilson and McPhaul, 1994). The AR-A isoform is from the same gene as AR-B but its N–terminal portion has been truncated due to an alternative translation initiation site, which starts with methionine 188 (Wilson and McPhaul, 1996).

Like all other steroid hormone receptors there are several distinct regions in the AR. The DNA-binding domain (DBD) is responsible for nuclear localization and interaction with hormone responsive elements and is comprised of 70 amino acids (Roy and Chaterjee, 1995). The amino acid sequences of this domain are similar among different steroid receptors (56-79%) and are identical in ARs from a variety of mammals (Hiipakka and Liao, 1998). The N–terminal domain is comprised of 555 amino acids and the AF-1 transactivation domain is situated in this region (Chang et al., 1988). An uncommon feature of this region in the human AR is the presence of polyglutamine (6-39 residues) and polyglycine (3-18 residues) repeats. It has been shown that the expansion of the polyglutamine repeats to 40-65 residues is associated with the X-linked spinal/bulbar muscular atrophy (SBMA) (LaSpada et al., 1991). SBMA is associated with partial androgen insensitivity, since the length and location of CAG trinucleotide repeats in the AR N–terminal domain affects transactivation function (Chamberlin et al., 1994). The ligand binding region (LBD) is located at the carboxyl terminal portion of the receptor and contains the transactivation domain AF-2, which regulates ligand-dependent receptor function (Roy and Charterjee, 1995). The human AR LBD contains 290 amino acids and is identical among humans, mice and rat ARs (Hiipakka and Liao, 1998). The AR in an inactive state is associated with heat shock proteins such as HSP70 and HSP90 through the LBD; this complex is disrupted after AR binding of androgens (Janne et al., 1993). The LBD also is required for dimerization of the receptors after ligand binding (Roy and Chaterjee, 1995).

Mechanism of Action

Androgens play an important role in the differentiation and development of the male sexual organs (Wilson et al., 1981). The AR has been implicated in physiological disorders such as partial and complete androgen insensitivity syndromes (Patterson et al., 1994), and the neoplastic transformation of the prostate (Cunha et al., 1987).

The action of AR in the cell is mediated by both genomic and non-genomic processes. The classical model of AR action is the positive and negative regulation of genes in a ligand-specific manner by binding to its response element (Beato, 1989). This response element can also be bound by progesterone, mineralocorticoid and glucocorticoid receptors (Cato et al., 1987; Ham et al., 1988). AR can also dimerize with glucocorticoid receptors and form heterodimers, however, this heterodimer blocks the transactivation function of the AR homodimer (Chen et al., 1997). The AR homodimer is able to activate transcription by binding to basal transcription factors through its N–terminal domain (McEwan and Gustafsson, 1997). The AR complex associates with coactivators such as transcriptional mediator/intermediary factor (TIF2), androgen receptor-associated protein (ARA70) and CBP (Voegel et al., 1996; Yeh and Chang, 1996; Aarnisalo et al., 1998).

The AR is not only involved in positive regulation of gene expression but also in negative regulation (Persson et al., 1990). One example of negative regulation is with the maspin gene in prostate cells. The expression of this gene is regulated positively by a ETS (Erythroblastoma twenty-six) element and

negatively regulated by an element site recognized by AR (Zhang et al., 1997). AR can negatively regulate the expression of the glycoprotein α-subunit by binding basal elements at the promoter region of this gene (Heckert et al., 1997). AR dimer can interact with c-Jun to inhibit the DNA-binding activity of this transcription factor (Kallio et al., 1995). AR also can interact with ETS proteins which are required for transactivation of the collagenase I gene in humans, resulting in the down-regulation of collagenase gene expression (Heckert et al., 1997). In the latter, the N–terminal of the AR is crucial for this activity (Gast et al., 1998). Another example of gene down-regulation by AR is through an indirect effect on interleukin-6 (IL-6). AR increases the expression of the inhibitor protein IκBα which sequesters NF-κB in an inactive form so that IL-6 expression is not stimulated (Keller et al., 1996). Interestingly, down-regulation of IL-6 gene expression involves IL-6 up-regulation of AR gene expression (Lin et al., 2001).

Another level of regulation of AR activity is through cross talk with other signal transduction pathways. It has been shown that the IL-6 signaling pathway is augmented by interaction of AR and STAT3 proteins, the latter acting as signal transducers of this pathway. Even more interesting is that augmentation of STAT3 activity occurs via physical interactions between AR and STAT3 (Matsuda et al., 2001). EGF and IGF-I can also enhance ligand-dependent and ligand-independent transactivation of AR (Reinikainen et al., 1996). The HER2/Neu is a transmembrane protein that is able to induce ligand-independent transactivation of AR through a MAP kinase pathway (Yeh et al., 1999). Modulator of protein kinase A (PKA) such as forkskolin can activate AR,

independent of its ligand (Nazareth and Weigel, 1996) or potentiate the ligand-dependent transactivation function of the AR (Ikonen et al., 1994). Also, stimulators of protein kinase C (PKC) can enhance ligand-dependent transactivation of AR (de Ruiter et al., 1996). Interestingly, the AR appears to be also involved in non-genomic effects since intracellular calcium concentration increases within two minutes of treatment of prostate cancer cells with dihydrotestosterone (Steinsapir et al., 1991). In another study, the concentration of intracellular calcium was increased within five to ten seconds after treatment of rat osteoblast cells with testosterone (Liebeherr and Grosse, 1994). In the mouse IC-21 macrophage cell line, which lacks classical AR expression, specific non-genomic responses to testosterone were observed (Benten et al., 1999). This was demonstrated by the finding of a rapid increase in intracellular free calcium after exposure to testosterone, which was mediated by a G-protein-coupled receptor for testosterone located in the plasma membrane (Benten et al., 1999).

Androgen Receptor in the Female Reproductive Tract

Androgens are abundant in circulation and in uterine tissues (Bonney et al., 1984; Stone and Seamark, 1985). Similarly, androgen binding sites have been detected in uterine tissues (Heyns et al., 1976). The AR, like ERs and PRs are present in the nucleus of both stromal and epithelial cells in human cycling endometrium (Horie et al., 1992). AR has also been detected in myometrial

smooth cells (Mertens et al., 1996). The AR has been shown to be functional in human endometrial cells in vitro, stimulating prolactin secretion (Narukawa et al., 1994). In ovariectomized rats treated with 5α-dihydrotestosterone (DHT), IGF-I mRNA was increased 20-fold relative to control. Although estrogen was able to similarly increase IGF-I mRNA levels, the mechanism used by each steroid hormone to induce IGF-I gene expression appeared different. The levels of IGF-I mRNAs stayed high for as long as a week after treatment with DHT, in contrast, estrogen produced only a transient increase (Sahlin et al, 1994). It is important to note that the increase in IGF-I by testosterone is not due to aromatization to estrogen, since the same effect was seen with DHT.

Elevation of systemic androgens have been associated with uterine hyperplasia or neoplasia in humans (Vitaratos et al., 1990). AR mRNA levels are increased by a treatment combination of estradiol and testosterone, however, estradiol alone had little effect on AR gene expression in the primate uterus (Adesanya-Famuyima et al., 1999). Using a human endometrial adenocarcinoma cell line (Ishikawa), Lovely et al. (2000) showed that AR is present in these cells and that its gene expression is increased by estradiol, while progesterone elicited an opposite effect. Another important observation from this cell line is that DHT and testosterone have a modest positive effect on AR gene expression. However, the crucial observation in these endometrial cells is that testosterone and DHT display an anti-estrogen effect by decreasing the expression of an estrogen-regulated gene, namely that of alkaline phosphatase.

Additionally, AR is negatively regulated by progesterone, and the use of RU486, a progesterone antagonist, increased AR expression in the glandular epithelium and in stroma cells of human endometrium (Slayden et al., 2001).

AR is expressed in most primate ovarian cells, including oocytes, granulosa, thecal, stromal and luteal cells (Hild-Petito et al., 1991; Tetsuka and Hillier, 1996; Duffy et al., 1999). The action of androgens on the follicle has been shown to enhance FSH-mediated effects such as FSH-stimulated progesterone and estrogen production (Hillier and de Zwart, 1982). Androgen stimulated the production of inhibin in cultured human (Tsonis et al., 1987) and bovine (Henderson and Franchimont, 1981) granulosa cells. The effect of androgens over FSH-stimulated granulosa cell differentiation is mediated by AR, since the use of the anti-androgen hydroxyflutamide, which inhibits nuclear translocation of AR, has been shown to block granulosa cell differentiation (Hillier and de Zwart, 1982). FSH induction of cAMP production in rat granulosa cells was also enhanced by pre-incubation with testosterone and DHT for 36 hours (Daniel and Armstrong, 1984).

Androgens are also implicated in promoting growth of ovarian tissues, since short-term androgen treatment stimulates growth of immature follicles, thecal hyperplasia and capsular thickness in primates (Vendola et al., 1998). Furthermore, androgen treatment increased the recruitment of primordial follicles into the growth pool, an effect correlated with an increase in IGF-I and IGF-I receptor gene expression in primordial oocytes in primates (Vendola et al.,

1999a). Treatments with testosterone and DHT also resulted in three- to four-fold increases in IGF-I concentration in granulosa, thecal and interstitial compartments; likewise, both androgens increased IGF-I receptors in theca cells and less markedly in granulosa and interstitial cells (Vendola et al., 1999b). Androgens have also been implicated as inhibitors of follicular development. Androgen treatment has been shown to inhibit LH receptor induction in rat granulosa cells (Jia et al., 1993). The selective down-regulation of P450 aromatase and of FSH and LH receptor mRNA expression are associated with granulosa cell apoptosis (Tilly et al., 1992). Androgens enhanced apoptosis of granulosa cells, although this was prevented by co-treatment with estrogen (Billig et al., 1993). Androgens also enhanced the activity and gene expression of tissue-type plasminogen activator in cultured rat granulosa cells previously exposed to gonadotropin-releasing hormone (GnRH) and epidermal growth factor (EGF) (Jia et al., 1990).

In rat granulosa cells, regulation of AR is under the control of FSH and androgens. This is supported by the fact that treatment of immature rat granulosa cells with androgens down-regulated AR mRNA levels and this effect can be reversed by co-treatment with FSH (Tetsuka and Hillier, 1996). AR is also expressed in primate mammary glands, where testosterone inhibited estrogen-induced ERα gene expression and cell proliferation in epithelial cells (Zhou et al., 2000).

Aromatase

Estrogen biosynthesis is catalyzed by the enzyme complex aromatase (Thompson and Siiteri, 1974). This enzyme complex is formed by two components: aromatase cytochrome P450 (P450 aromatase) and a flavoprotein NADPH-cytochrome P450 reductase (Simmons et al., 1985). The P450 aromatase is a member of the cytochrome P450 superfamily gene, which currently contains over 300 members in some 36 genes. The P450 aromatase is the only member of the gene family 19, designated CYP19 family (Nelson et al., 1993). This nomenclature is based on the mechanism by which oxygen attacks the carbon of the methyl group at position 19. This heme protein is responsible for removing the 19-carbon steroid substrate and catalyzing several reactions that lead to the formation of the phenolic A ring characteristic of estrogens (Simpson et al., 1994). The typical cytochrome P450 enzyme incorporates molecular oxygen into substrates by reactions that are dependent on efficient electron transfer from donor molecules. During the process of androgen aromatization, it is required that there is a sequential transfer of three pairs of electrons and the consumption of three moles of oxygen and three moles of reduced NADPH to produce one mole of estrogen (Thompson and Siiteri, 1974). Lysine and histidine in the catalytic site of the P450 aromatase are required for final formation of the A ring in the estrogen molecule (Korzekwa et al., 1993).

Androstenedione and testosterone are the most common, well-recognized

and probably the most physiologically important substrates for P450 aromatase (Conley and Hinshelwood, 2001). The 16-hydroxy-androstenedione produced by hepatic hydroxylation of fetal adrenal dehydroepiandrosterone sulphate (DHEAS) is another substrate used by the placental P450 aromatase to produce estriol (Siiteri et al., 1982). Normally androgens are produced by male testes, female ovaries and placenta; the adrenal cortex can also produce androgens (Norman and Litwack, 1997). In the case of postmenopausal women, the principal source of androgens is the adrenal cortex, which produces androstenedione, dehydro-epiandrosterone (DHEA), and DHEAS (Labrie et al., 1997). However, DHEA has to be converted to androstenedione first in order to be used for estrogen production. This conversion is catalyzed by 17-hydroxysteroid dehydrogenase, which is found in several tissues such as tumor breast epithelium (Sasano et al., 1996), bone (Sasano et al., 1997) and adipose tissues (Corbould et al., 1998).

P450 aromatase is located in the microsomal environment of the endoplasmic reticulum (Corbin et al., 2000). Placental P450 aromatase can also be associated with the mitochondrial compartment (Fournet-Dulgueron et al., 1987). It has been reported that the aromatase complex and 17α-hydroxylase are associated with the plasma membrane, and indeed, the plasma membrane is another subcellular fraction that exhibits aromatase activity (Amarneh and Simpson, 1996). Analyses of ovarian follicular tissues indicate that P450 aromatase expression is tightly correlated with the expression of 17α-hydroxylase (Fortune, 1994). In the two-cell theory of steroidogenesis, theca

interna cells express 17α-hydroxylase and produce androgens, which are then converted in the granulosa cells expressing P450 aromatase (Hillier et al., 1994). This system of compartmental separation of androgen and estrogen synthesis also occurs during pregnancy, where fetal adrenal androgens are metabolized to estrogen in the placenta (Norman and Litwack, 1997). Another example of compartmentalization is in pregnant rats where placental androgens are aromatized in maternal ovaries (Hickey et al., 1988).

P450 aromatase does not always complete the aromatization of androgen to estrogen, and several by-products are formed. Some of these products are 19-hydroxy-androstenedione, 19-oxo-androstenedione and 19-oxo-testosterone, all of which are generated from cells in the placenta and gonads (Corbin et al., 2000). The production of these intermediatary metabolites has been related to low expression of reductase, which cannot saturate P450 aromatase (Swinney et al., 1993). In humans, the levels of 19-hydroxy-androstenedione increase faster than those of estrogen as pregnancy progresses (Osawa et al., 1990). In other species like the horse, the placenta can produce 19-nortestosterone and this production can be blocked in vitro by the use of aromatase inhibitors (Moslemi et al., 1995). In pigs, there was an early report showing production of 19-norandrogens in granulosa cells by P450 aromatase (Khalil et al., 1988). In a recent study conducted by Kao et al. (2000) using two isoenzymes of porcine P450 aromatase (placental and embryonic type), it was demonstrated that the major products of the placental type were 17β-estradiol and 19-nortestosterone

at a ratio of 94:6. However, the embryonic ratio of these compounds was found to be 6:94, suggesting that the major metabolite produced by embryonic P450 aromatase is 19-nortestosterone. P450 aromatase also exhibits other catalytic activities including that of 2-hydroxylase, which produces some catechol estrogens in the human and equine placenta (Osawa et al., 1993; Almadhidi et al., 1996).

Gene Structure

The human P450 aromatase protein has been well characterized and is encoded by a single gene (Simpson et al., 1994). The size of the human P450 aromatase gene is around 75 kb. It is composed of 10 exons with the coding region formed from exons II to X (Means et al., 1989; Harada et al., 1990). The protein encoded by the human P450 aromatase contains 503 amino acids (Corbin et al., 1988). There are multiple first exons that are involved in tissue-specific expression, and these are untranslated (Means et al., 1991; Jenkins et al., 1993). The untranslated exon I.1 in the human placenta isoform is located around 40 kb upstream from the translation start site in exon II (Means et al., 1991). However, the ovary isoform untranslated exon I is located close to exon II (Jenkins et al., 1993). The adipose tissue isoform contains the untranslated exon I.4, which is located 20 kb upstream from exon I.1 (Mahendroo et al., 1993). Since these untranslated exons are not part of the coding sequence the protein coded from those mRNA are identical (Simpson et al., 1997).

The bovine P450 aromatase gene was mapped to a band q2.6 of chromosome 10 and shown to produce a protein of 503 amino acids in length (Hinshelwood et al., 1993; Goldammer et al., 1994). The first exon of the bovine P450 aromatase is located 19 kb upstream from the start site of translation (Brunner et al., 1998). A different isoform has been obtained from bovine placenta, and shown to contain sequences for exons II, III, V, VIII, and IX. In addition, the exon IV segment was replaced by a stretch of repeated sequences, exons VI and VII were absent, and several stop codons existed within the open reading frame, suggesting the presence of a bovine pseudogene (Fürbass and Vanselow, 1995). In porcine, the P450 aromatase gene undergoes a duplication process (Choi et al., 1996; Graddy et al., 2000). The porcine genome contains three different transcripts that encode distinct isozymes. They have been cloned from the gonads (Corbin et al., 1995), placenta, and conceptus trophoblastic cells (Choi et al., 1996). Each gene has differences within the coding regions, which result in a number of amino acid changes (Graddy et al., 2000). Besides these three genes, the trophoblastic isozyme contains two different exon I (IA and IB), which are located 8 kb upstream from the start site of translation (Choi et al., 1996). The same duplication has been reported in fish, with each gene encoding slightly different proteins (Tchoudakova and Callard, 1998).

Gene Expression and Regulation

Aromatase expression is widely distributed throughout the body. In most mammals studied, aromatase expression occurs in the gonads and in the brain. In humans, P450 aromatase is expressed in the pre-ovulatory follicles and corpora lutea (Means et al., 1991). Also, expression of P450 aromatase is much higher in the granulosa cells of preovulatory follicles than in small follicles (Hickey et al., 1988). In the testes, aromatase activity is present in Sertoli cells before puberty and in Leydig cells of adult male rodents (Abney, 1999). In bovine, P450 aromatase has been reported to be highly expressed by granulosa cells and placenta; expression was also detected in testis, brain, adrenal glands, and liver but at lower levels (Fürbass et al., 1997). In mouse, P450 aromatase activity and transcripts have been detected in testicular germ cells, elongating spermatids and epididymal spermatozoa (Janulis et al., 1996). In rat brain, high levels of P450 aromatase are present in the medial basal hypothalamic area and amygdaloid region (Lephart, 1996).

In addition to gonads and brain, other tissues also express P450 aromatase. In humans, P450 aromatase is also found in placental syncytiotrophoblasts, adipose tissue, vascular system, fetal liver, skin fibroblast and bone osteoblast (Simpson et al., 1994; Harada et al., 1999). Simpson et al. (1994) reported that the wide expression of P450 aromatase gene in humans is regulated by tissue specific promoters and by alternative splicing. For example, in the ovarian granulosa cells, P450 aromatase expression is primarily under the

control of FSH, whose action is mediated through cAMP (Michael et al., 1997). The elevated expression of P450 aromatase in granulosa cells from preovulatory follicle is dramatically decreased in vivo by the surge of LH that occurs at the time of ovulation (Hickey et al., 1988); a similar response is obtained when cultured granulosa cells receive LH (Hickey et al., 1990). Steroidogenic factor-1 (SF-1) and CREB have been shown to bind the human P450 aromatase promoter region and stimulate transcription (Michael et al., 1997). In the human placenta, the expression of P450 aromatase is under the control of a strong promoter I.1 upstream of untranslated exon I.1, which is located 40 kb upstream from exon II (Means et al., 1991). It has been suggested that the factor responsible for human placental P450 aromatase expression is retinoic acid (Simpson et al., 1997).

P450 aromatase expression has been found in porcine placenta and embryonic trophoblastic cells (Choi et al., 1996), and a developmental switch occurs between the embryonic P450 aromatase type to the placenta type with placental maturation (Choi et al., 1997). Also, Graddy et al. (2000) reported a switch from porcine embryonic P450 aromatase expressed in the endometrium to the placental type between days 30 to 60 of gestation. A great difference in catalytic activity has been reported between the placental and embryonic types (Kao et al., 2000) suggesting that the switch in expression during pregnancy could be due to a different requirement for estrogen production. Studies in pigs have confirmed that P450 aromatase is also expressed in the theca interna cells,

in addition to granulosa cells, of preovulatory follicles (Conley et al., 1997).

In human adipose tissues, the expression of P450 aromatase is driven by a distal promoter I.4, and the transcripts contain primarily the untranslated exon I.4 at their 5' end (Mahendroo et al., 1993). The promoter region of the untranslated exon I.4 contains a TATA-less promoter, as well as an upstream glucocorticoid response element (GRE) and an Sp1 sequence, both of which are required for the expression of the transcript in the presence of serum and glucocorticoid (Zhao et al., 1995a). These authors also found that this region contains an interferon-γ activating element (GAS). Culture of preadipocytes treated with glucocorticoids or serum stimulated expression of P450 aromatase (Simpson et al., 1997). The stimulatory effect produced by serum on P450 aromatase expression in these preadipocyte cells can be mimicked by class I cytokines such as interleukin-11 (IL-11), IL-6, oncostatin, and leukaemia inhibitory factor (LIF) (Zhao et al., 1995b). Treatment of adipose stromal cells with these cytokines resulted in a rapid phosphorylation of Jak-1 kinase, and this action resulted in rapid phosphorylation of STAT-3 protein (Zhao et al., 1995b). Tumor necrosis factor α (TNF-α) also stimulated P450 aromatase expression in the adipose stromal cells in the presence of dexamethasone. This effect of TNF-α uses an AP-1 site upstream of the GAS element within the promoter I.4 (Zhao et al., 1996). In human breast carcinomas, there is a switch in promoter usage in the P450 aromatase gene. Transcripts from normal breast tissue contain the untranslated exon I.4, however, transcripts from a breast carcinoma tumor

contains primarily sequences that are immediately upstream of exon II (Sasano and Harada, 1998).

Mammalian embryos have been reported to express P450 aromatase. Human embryos have been shown to produce estradiol (Edgar et al., 1993). Also high aromatase activity have been detected in porcine embryos (Green et al., 1995), and this embryonic isoform is produced by a unique gene, which is completely different from the other porcine isoform genes (Choi et al., 1996; Graddy et al., 2000). Camel blastocysts also exhibit high levels of P450 aromatase activity to produce 17β estradiol (Skidmore et al., 1994). Equine pre-attachment blastocysts express P450 aromatase in the trophectoderm cells (Choi et al., 1997; Walters et al., 2000). The significance of estrogen production by these mammalian embryos is still unknown, with the exception of the estrogens produced by porcine embryos. In this species, estrogens are considered to function as signals for maternal recognition of pregnancy (Bazer et al., 1984).

Early Gestation in the Pig

Embryo Development

The pig is a polytococous species with a high incidence of prenatal mortality. The percentage of embryo mortality averages between 20-30% before

day 30 of gestation, with another 10 % occurring between day 40 to term (Pope and First, 1985). The majority of embryonic mortality occurs during the time of periimplantation. During this period the conceptus produces and secretes estrogens(Gadsby et al., 1980; Pusateri et al., 1990), the proposed signal for maternal recognition of pregnancy. Porcine conceptuses also undergo dramatic changes in morphology going from a spherical conceptus (10 mm in diameter) to tubular (20-40 mm in length) and ultimately filamentous (100 mm in length) forms in less than 3 to 4 h (Geisert et al., 1982). Several investigators have postulated that this change in morphology is the result of cellular remodeling instead of cell proliferation (Geisert et al., 1982; Mattson et al., 1990). However, a recent experiment (Ka et al., 2001) demonstrated intensive nuclear staining for proliferative cell nuclear antigen protein (PCNA) in all the trophectoderm cells of day 12 filamentous conceptus by the time of elongation. This data suggest that day 12 trophectoderm cells from filamentous conceptuses are actively dividing.

Previous to the time of maternal recognition of pregnancy on day 7 to 8, the blastocyst is composed of an outer layer of polarized trophectoderm, an embryonic disc, and an inner layer of endodermal cells (Stroband and Van der Lende, 1990). On day 10, the conceptus is a small sphere of 1 to 3 mm in diameter, and the sphere expands 3 to 8 mm in diameter over the next 30 hours (Geisert et al., 1982). Once the conceptus reaches 10 mm in diameter on day 11, it undergoes a transformation to filamentous morphology. The first signal for the appearance of the third embryonic layer (mesoderm) is detected in embryos

5 to 6 mm in diameter (Stromband and Van der Lende, 1990). Also, the expression of brachyury, a transcription factor involved in mesoderm formation is first detected in 6 mm spherical embryos (Yelich et al., 1997). The appearance of the mesoderm is coincident with the expression of P450 aromatase and P450 17α-hydroxylase genes (Green et al., 1995; Yelich et al., 1997) and the capacity of the conceptus to synthesize estrogen (Pusateri et al., 1990). Although the expression of these two enzymes are low, their expression starts to increase as the conceptus continues to grow, reaching a peak of maximum expression at the tubular stage, a decrease in expression on day 12 filamentous, and become almost undetectable on day 14 filamentous (Green et al., 1995).

Porcine conceptuses also express growth factors and growth factor receptors (Vaughan et al., 1992; Green et al., 1995). IGF-I is present in uterine luminal fluids at peri-implantation, and maximum levels occur during the period of conceptus elongation (Simmen et al., 1992). The increase in estrogen synthesis during this period is due to the stimulation of P450 aromatase activity and/or gene expression by IGF-I (Hofig et al., 1991; Green et al.,1995). Biological activity of IGF-I is regulated in tissues not only through its synthesis, but also by the presence or absence of IGF-binding proteins (IGFBPs) (Rechler, 1993). Gene expression for IGFBP-2 and -6 have been detected in porcine endometrium (Simmen et al., 1992; Song et al., 1996), and IGFBP-2 and -3 proteins have been detected in the uterine flushes (Lee et al., 1998). It has been proposed by Lee et al. (1998) that luminal protease activity is induced by

developing conceptuses, which leads to a cleavage of the IGFBPs and resulting in the increase of IGF-I bioavailability for the embryo. Other growth factor receptors such as epidermal growth factor (EGF) receptors have been detected in pre-elongated conceptuses (Vaughan et al., 1992). EGF receptor bind transforming growth factor α (TGF-α), and TGF-α mRNA levels in developing conceptuses peak at the time of conceptus elongation, with expression declining after conceptus elongation (Vaughan et al., 1992). Porcine conceptuses also express keratinocyte growth factor (KGF) receptor or fibroblast growth factor-7 (FGF-7) receptor, also called FGF receptor 2IIIb (Ka et al., 2000). KGF expression in the uterine epithelium is stimulated by conceptus estrogens, and KGF produced and secreted by these cells act on the porcine conceptus to stimulate their proliferation and differentiation (Ka et al., 2001)

Members of the TGFβ superfamily are also expressed by porcine conceptuses (Gupta et al., 1998a;b). Gupta et al. (1998a) showed that TGFβ-1, 2 and 3 mRNA are present in the embryonic trophectoderm, mesoderm and endoderm, and their expression increases 2 to 4 fold in the trophectoderm and endoderm layers between days 10 and 14. The presence of the three TGF-β types (1, 2 and 3) was also detected in the uterine luminal fluids, and receptors for TGFβ-I and β-II were present in the uterine luminal epithelium (Gupta et al., 1998b). These results suggest autocrine and paracrine actions of these proteins.

Porcine conceptuses also express cytokines which may regulate immune

function within the porcine uterus. Interleukin 1β (IL-1β) is expressed by porcine conceptuses during the time of maternal recognition of pregnancy (Tuo et al., 1996). LIF-receptor, but not LIF is expressed by conceptuses during peri-implantation (Yelich et al., 1997; Modric et al., 2000). Interestingly, IL-6 is only transiently expressed by day 12 filamentous conceptuses (Modric et al., 2000). Porcine trophectoderm cells express high levels of interferon gamma (IFN-γ), which has antiviral effect in the pregnant pig uterus during peri-implantation (Lefevre et al., 1990). Porcine trophectoderm also secretes a newly discovered interferon called IFN-δ, which is coexpressed with IFN-γ (Lefevre, 1998).

Another factor involved in porcine embryo development is retinol. Uterine fluids contain high levels of retinoic acids (Trout et al., 1992). The porcine embryo has been demonstrated to express retinol binding protein (RBP) (Harney et al., 1990), as well as retinoic acid receptor α (RARα) and RARγ mRNA and proteins (Harney et al., 1994). The RARα, β and γ are present in the conceptus before elongation (Yelich et al., 1997). Since retinoic acid can influence the production of several extracellular matrix components (Schule et al., 1990) and cell surface adhesive molecules (Agura et al., 1992), it has been postulated that RBP and RAR may have a role in the initiation and/or eventual remodeling of the trophoblast during conceptus elongation (Geisert and Yelich, 1997). Additionally, retinol can cause embryo toxicity at elevated concentrations (Thompson et al., 1964). An increase in retinol levels in the uterine lumen as a consequence of an increase in blood flow to the uterus at day 12 of pregnancy

(Trout et al., 1992), is likely due to the action of estrogens produced by developing embryos (Ford et al., 1982). Roberts et al. (1993) proposed that the high rates of embryo mortality at early gestation could be due to the high levels of retinol caused by estrogen secretion from the more developed embryos, which are toxic to less developed embryos. Related to this, it has been postulated that embryo asynchrony is a major cause of embryo mortality, and this is directly correlated to the capacity of large embryos to secrete estrogens and affect the survival of less developed embryos (Ford, 1997). Wilson and Ford (1997) showed that in a porcine Chinese breed (Meishan) characterized by a large litter size, embryos grow in more synchrony than those of European breeds, as well as produce less estrogen. All these results suggest that estrogen could be a key factor in embryo mortality.

Implantation

The process of implantation also known as a receptive stage of the uterus occurs after maternal recognition of pregnancy, which is characterized by conceptus migration, spacing, elongation, and production of copious amounts of estrogen in the pig (Geisert et al., 1982; Pusateri et al., 1990). The porcine conceptus does not normally invade the uterine epithelium. However, it has the ability to invade non-uterine epithelium and form a syncytium when placed in an ectopic site (Samuel and Perry, 1972). During the pre-receptive stage, the conceptus cannot attach to the uterine epithelium. Uterine epithelial cells avoid

attachment of the conceptus by exposing abundant negatively charged apical glycocalyx in its uterine luminal surface (Dantzer, 1985). Consistent with this, the glycocalyx of the uterine epithelium is reduced at the implantation site during initial attachment (Blair et al., 1991). There are several factors that are likely responsible for the pre-receptive stage of the porcine endometrium, such as mucin and C-CAM (Cell-Cell Adhesion Molecule) (Aplin, 1997). Porcine luminal epithelium expresses a transmembrane glycoprotein that is heavily glycosylated (200-1000 kDa) known as MUC-1 (Mucin gene 1)(Bowen et al., 1996). It can function as an anti-adhesive molecule by sterically inhibiting adhesive molecules expressed at the conceptus from adhering to the uterine surface (Carson et al., 1998). MUC-1 expression studies in the uterine endometrial epithelium have shown that the protein is present on day 8 but not on days 10 to 15 in pregnant pigs, and the decrease in MUC-1 expression is correlated with an increase in progesterone concentration (Bowen et al., 1996).

For implantation to begin in the pig, the uterine luminal epithelium is transformed from a columnar epithelium with microvilli during the pre-receptive stage to an epithelium with dome-like apical surface devoid of microvilli (Keys and King, 1990). Along with these changes, there is an increase in progesterone in the uterine lumen and a decrease in epithelial cell permeability (Johnson et al., 1988). The uterine epithelial cells also begin to express several adhesion molecules such as integrins which are involved in the attachment of the porcine conceptus (Burghardt et al., 1997). The integrin subunit molecules identified to

be expressed by the uterine epithelium during early pregnancy include α-1, α-3, α-4, and αv . The conceptus also expresses the same integrin subunits (Bowen et al., 1996). The extracellular matrix ligands for these integrins include vitronectin and fibronectin, which are also expressed by both uterine epithelium and conceptus (Browen et al., 1996).

Another glycoprotein expressed by the uterus during the time of implantation is a 30 kDa basic endometrial glycoprotein (GP30), which is a cleavage product of a 120 kDa multi-functional inter-trypsin inhibitor heavy chain-like protein (IαIH4) (Geisert et al., 1995). The IαIH4 protein is a substrate of Kallikrein enzyme, whose activity increases by day 12 of pregnancy in the uterus (Vonnahme et al., 1999). Changes in carbohydrate expression on the uterine epithelium also accompany porcine implantation. The expression of the blood group determinant H-type-1 antigen, which is a carbohydrate, is high during the first 4 days after conception and its expression declines between days 8 to 15 during pregnancy (Burghardt et al., 1997). Another carbohydrate antigen localized in the porcine uterine epithelium from days 14 to 30 of pregnancy is Lewis X determinant, which is a ligand for E-selectin (Whyte and Binns, 1994).

The proposed mechanism of implantation by Burghardt et al. (1997) consists of four phases. The first phase begins when progesterone induces changes in the uterine epithelium, resulting in loss of apical microvilli, and increased close contact between conceptus and apical uterine epithelium. The second phase is characterized by initial interactions between the embryonic

trophectoderm cells and the luminal epithelial cells through transmembrane molecules that are either induced or constitutively expressed but are otherwise unavailable until the loss of MUC-1. The third phase represents the beginning of signal transductions from transmembrane molecules, and the last phase is the final adhesion event which is mediated by high-affinity integrin-ligand interactions. The final outcome of the highly orchestrated process is the attachment of the conceptus to the uterine epithelium.

CHAPTER 3
MOLECULAR CLONING OF PORCINE ESTROGEN RECEPTOR β cDNAs AND DEVELOPMENTAL EXPRESSION IN PERI-IMPLANTATION EMBRYOS

Introduction

Among mammals, the pig is one species in which the embryo produces copious amounts of estrogens (E) in a manner dependent on its stage of development (Gadsby et al., 1980; Pusateri et al., 1990). In this tissue, E synthesis is largely regulated at the levels of cytochrome P450 aromatase (P450 arom) and 17α-hydroxylase, the last two enzymes in the E biosynthetic pathway (Conley et al., 1992; Green et al., 1995). Peak levels of embryonic E occur around days (d) 11 and 12, a period correlated with maternal recognition of pregnancy (Flint et al., 1979). The main target of this E is likely the endometrium, which exhibits functional E receptors (ER) (Geisert et al., 1993) and which, upon E and progesterone (P) stimulation, secretes a myriad of growth factors, cytokines, proteases, protease inhibitors, and other yet unknown molecules, that are requisite for implantation and pregnancy success (Roberts et al., 1993; Simmen et al., 1995).

The biological effects of E are mediated by two ER subtypes, ERα and

ERβ (Petterson and Gustafson, 2001). While the distribution of both ER isoforms has been widely documented in female reproductive tissues of numerous mammalian species, and their respective functions have been inferred from the use of specific ERα and ERβ knockout mouse models (Couse and Korach, 1999), studies that address the embryo as a direct target for E action via ER-mediated mechanisms are limited (Hou and Gorski, 1993; Hou et al., 1996; Ying et al., 2000) and in some cases, conflicting (Yelich et al., 1997; Dekaney et al., 1998). Moreover, the functionality of the reported embryonic ER has not been extensively evaluated, specifically with respect to the molecular mechanisms underlying their potential regulatory roles in embryonic growth and/or development.

The pig, like the human, suffers from high rates (30-40%) of peri-implantation embryo mortality (Pope and First, 1985). The cause for this condition in humans is not well-understood; in the pig, however, the asynchronous development of embryos whereby the larger, more developed embryos (tubular, T; filamentous, F) impair the development of their smaller (spherical, S) counterparts has been postulated to underlie embryonic death (Pope et al., 1990). The possibility that E is involved in this asynchrony was borne from the findings that E is toxic to porcine embryos when administered prior to the time of maternal recognition of pregnancy (Morgan et al., 1987). The more developed porcine embryos also exhibited greater E production than less developed counterparts (Pusateri et al., 1990; Green et al., 1995), and a line of pigs (Meishan) exhibiting increased prolificacy and greater within-litter synchrony

in embryo development displayed lower levels of E production than a line (Large White) that suffers from high embryo mortality (Wilson and Ford, 1997).

ER-mediated E action has long been implicated in the control of cell growth (Clark and Markaverich, 1988). Moreover, recent data have provided increasing support to the notion that positive- or negative-regulation of cell growth by E is a function, in part, of the ER subtype expressed in target cells (Iafrati et al., 1997; Lindner et al., 1998; Jarvinen et al., 2000; Kahlert et al., 2000). Thus, the identification of the predominant ER isoform in porcine developing embryos, if present, is necessary for determining whether and how E plays an essential embryotrophic role in early pregnancy events. Indeed, a finding of distinct expression of ERα and ERβ by porcine embryos at discrete developmental stages will likely provide important insights into possible molecular mechanisms whereby E may be correlated with embryonic asynchrony.

The major objective of the present studies was to evaluate if distinct ER isoforms are expressed by peri-implantation embryos. Towards this end, the sequence of the complete open reading frame of porcine embryo ERβ mRNA was determined. Further, the developmental expression of ERβ, relative to ERα, and in concert with those of PR and other genes (PR, P450 aromatase Type III, Cyclin D1) known to be involved in growth and differentiation, was evaluated in embryos of different morphology by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Finally, in vitro cultures of d12F embryos were used to examine the effects of E on ERβ and PCNA gene expression.

Materials and Methods

Tissue Collection and RNA Preparation

Porcine embryos were collected at d10, 12, and 14 of pregnancy and separated according to size (diameter) and morphology (S, T, F) as previously described (Green et al., 1995). Total cellular RNA was isolated from tissues using TRIzol reagent (Gibco BRL. Grand Island, NY) following the manufacturer's instructions.

Porcine ERβ cDNA Cloning and Nucleotide Sequence Analysis

The cDNA was transcribed from total RNA (5 μg) prepared from porcine d12F embryos following the manufacturer's protocol (cDNA Cycle kit; Invitrogen, Carlsbad, CA). The strategy used for PCR cloning is shown in Fig. 3-1. Several primer sets based on conserved sequences from rat, human, bovine and ovine ERβ mRNAs were synthesized (Gemini Biotech, The Woodlands, TX.), and employed for PCR amplification using an Eppendorf Mastercycler Gradient (Eppendorf Scientific Inc., Westborg, NY) under optimized $MgCl_2$ concentrations and pH (Table 3-1) in 1XPCR buffer (5X= 300 mM Tris-HCl, 75 mM ammonium sulfate). The PCR products were electrophoresed on 1.5% agarose gels, and the expected DNA fragments were subcloned into TOPO vector (Invitrogen). The nucleotide sequences of the generated clones were determined at the Nucleotide Sequencing Facility of the Interdisciplinary Center for Biotechnology Research of the University of Florida, and compared with those of other

FIG. 3-1. Cloning strategy for the isolation of porcine embryo ERβ cDNAs using RT-PCR. Relative position of specific primers (1 to 4; F, forward; R, reverse) are shown. Primers were designed within conserved regions of human and bovine ERβ mRNAs, and the nucleotide sequences of the overlapping cDNA clones were determined. The sizes of the PCR fragments obtained from each primer set are indicated.

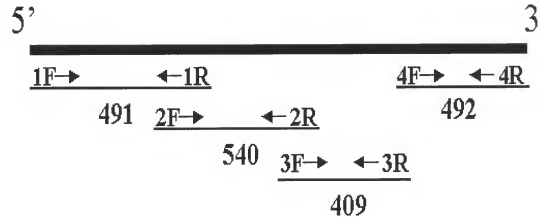

5'
3'
1F→
←1R
4F→
←4R
491
2F→
←2R
492
540
3F→
←3R
409

TABLE 3-1. Oligonucleotide primers and RT-PCR conditions for cloning porcine embryo ER-β cDNA coding region.

Primer Set		Primer Sequence	Size (bp)	Buffer 5X	Conditions °C	Time
1F	F	5'ATGGATATAAAAAACTCACC 3'	491	10 mM $MgCl_2$ pH 9.0	95 53 72	45 sec 1 min 1 min
1R	R	5' AGTACACTTCCTACATTCCG 3'			35 cycles	
2F	F	5' GGAAGGAAAAGTTACAGAGA 3'	540	17.5 mM $MgCl_2$ pH 8.5	95 51 72	45 sec 1 min 3 min
2R	R	5' CAGCTTGGTGAGGGACATCAT 3'			35 cycles	
3F	F	5' CTGGTGCTCACGCTCCTGGA 3'	409	17.5 mM $MgCl_2$ pH 9.5	95 51 72	45 sec 1 min 1 min
3R	R	5' CATGGCCTTGACACAGAGATA 3'			40 cycles	
4F	F	5' AGGGAAATGTGTAGAAGGAA 3'	492	10 mM $MgCl_2$ pH 9.0	95 62 72	45 sec 1 min 1 min
4R	R	5' CATTCAGCATCTCCAGCAGCA 3'			35 cycles	

F, Forward; R, reverse

mammalian ERβ sequences to confirm their identities and orientations relative to previously reported ERβ mRNA sequences (GenBank database). The porcine embryonic ERβ reported here has GenBank Accession Number AF164957.

Northern Blot Analysis

Total cellular RNA (30 μg) isolated from d12F embryos, and poly(A)+ RNA purified on oligo(dT) columns (Qiagen Inc.,Valencia, CA) from total cellular RNA prepared from d12 pregnant pig uterine endometrium were fractionated on a 1% agarose-formaldehyde-MOPs [3-N-morpholino-propanesulfonic acid] gel, and transferred onto a Biotrans nylon membrane (ICN Biotech., Irvine CA) by capillary transfer. The membrane was hybridized to nick-translated porcine ERβ cDNA (gel-purified, 491 bp insert from the 5' end of the porcine embryo ERβ mRNA; Fig.3-1) at 42 °C overnight in UltraHyb buffer (Ambion Inc., Austin, TX). After hybridization, the membrane was washed twice for 15 min each at 42°C in 2X saline sodium citrate (SSC), 0.1% SDS, and then in 0.1X SSC, 0.1% SDS for 30 min at the same temperature. The presence of ERβ transcripts was detected by autoradiography at -80°C. The sizes of the transcripts were calculated based on their migration positions relative to those of 18S and 28S ribosomal RNAs.

Immunolocalization of ERβ

Immunohistochemistry on d12 filamentous embryos using anti-ERβ antibody (Upstate Biotechnology, Lake Placid, NY.) was performed as previously described (Rosenfeld et al., 1999). In brief, d12F embryos were fixed in 4%

paraformaldehyde for 24 h and then processed and embedded in paraffin. Sections of 5 μm were cut, de-paraffinized, and subsequently re-hydrated through a series of alcohol dilutions. Sections were placed in 0.01 M citric acid (pH 6), and microwaved for 15 min to expose the cellular antigens (Shi et al., 1991). Nonspecific binding and endogenous peroxidase activity were blocked by incubation of sections in 1% hydrogen peroxide and then in normal horse serum (2% final concentration). The slides were then incubated overnight at 4°C with polyclonal anti-ER-β antibody (10 μg/ml; Upstate Biotechnology) generated in rabbits against the N-terminal region (amino acids 54 to 71;YAEPQKSPWCEARSLEHT) of the rat ERβ protein sequence (Byers et al., 1997). As negative controls, tissue sections were incubated with normal pre-immune IgG from rabbit. The biotinylated anti-rabbit (Biomeda Corp, Foster City, CA) IgGs was used as a secondary antibody. Sections were placed into a substrate solution of diaminobenzidine (Sigma, St Louis, MO.) for staining. Photo microscopy was performed using a Zeiss Axioplan 2 microscope with an RGB Spot Digital camera system with corresponding software (Carl Zeiss, Inc, New York, NY.)

RT-PCR Analysis of Embryo Gene Expression

The cDNA was synthesized from equal amounts of total RNA (5 μg) prepared from porcine embryos of different sizes and morphologies as previously described (Green et al., 1995). PCR amplification was performed in a total reaction volume of 50 μl containing 1μl of cDNA template, 50 pmol of the

forward and reverse primers (Table 3-2), 10 nM of each of the dNTPs, 1X PCR Buffer (Table 3-2) and 1 U of Taq polymerase (Boehringer Mannheim). The number of cycles used for each PCR reaction was optimized so that the amplification process was carried out within the linear range. All PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide, and were visualized under UV light. DNA fragments were quantified using an Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Co., San Leandro, CA).

Embryo In Vitro Culture

Porcine d12F embryos were preincubated in Medium 199 (M199) (GIBCO BRL) containing 1% antibiotic/antimycotic solution (ABAM) at 37° C in an atmosphere of N_2:O_2:CO_2 (50%:47.5%:2.5%) for 2 h, immediately after removal from the uterus. Medium was then replaced with fresh M199 containing vehicle alone (ethanol) or 17β-Estradiol (E_2; 50 nM final concentration; Sigma) in vehicle, and embryos were further incubated for 24 h under the same conditions. The embryos were then collected for RNA isolation and subsequent RT-PCR analysis.

Statistical Analysis

Data from in vitro embryo culture gene expression analysis (n = 5 experiments, where each experiment represents pooled embryos from a different d12 pregnant pig) were subjected to least squares ANOVA using the General

TABLE 3-2. Oligonucleotide primers and RT-PCR conditions for amplifications and detection of porcine embryonic genes.

mRNA		Primer Sequence	Size (bp)	Conditions °C	Time
ER-β	F	5' AGAGACATTGAAAAGGAAGG 3'	150	95 55	45 sec 2 min
	R	5' GCCTTACATCCTTCACATGA 3'		72	1 min
				35 cycles	
ER-α	F	5' ATTGGTCTTGTCTGGCGCTCC 3'	451	95 65	2 min 2 min
	R	5' GGTCATAGAGGGGCACCACGT 3'		72	3 min
				40 cycles	
Cyclin D1	F	5' CTGGCCATGAACTACCTGGA 3'	482	95 51	45 sec 45 sec
	R	5' GTCACACTTGATCACTCTGG 3'		72	3 min
				30 cycles	
P450Aromatase Type III	F	5' GCGCATGGTGACCGTCTGTG 3'	649	95 62	45 sec 1 min
	R	5' CATGACGAGGTCCACGACAG 3'		72	3 min
				25 cycles	
GAPDH	F	5' AGTATGATTCCACCCACGGC 3'	593	95 62	45 sec 1 min
	R	5' TCAGATCCACAACCGACACG 3'		72	3 min
				20 cycles	
PCNA	F	5' CTGCGGTCTGAGGGCTTCGA 3'	520	95 47	45 sec 45 sec
	R	5' ACCGTTGAAGAGAGTGGAGT 3'		72	1 min
				35 cycles	

F, Forward; R, reverse

Linear Models procedures of the Statistical Analysis System (Barr et al., 1979). Values were considered significant at $p < 0.05$ and are presented as least squares means (LSM) ± SEM

Results

Molecular Cloning and Nucleotide Sequence Analysis of Porcine Embryonic ERβ cDNAs

Analysis of the published nucleotide sequences of ERβ mRNAs from human (Mosselman et al., 1996), rat (Kuiper et al., 1996), mouse (Tremblay et al., 1997), ovine (Accession no.AF177936) and bovine (Rosenfeld et al., 1999) allowed the design of multiple primer sets within conserved regions, which were then used to generate overlapping DNA fragments utilizing porcine d12F embryo RNAs as template for RT-PCR. The four overlapping fragments of variable sizes (491, 540, 408 and 492 bp) (Fig 3-1) were sequenced. The composite sequence of 1581 nucleotides (nt) encode a 526 amino acid protein representing porcine ERβ. Alignment of this deduced amino acid sequence with those reported for other mammalian species revealed an overall identity, on average, of greater than 90% (Fig. 3-2). The highest identity of the porcine ERβ was observed to the bovine and ovine homologs (92 and 91%, respectively), with lesser identity demonstrated to human (86%), rat (87%), and mouse (86%) proteins. The N-terminal domain (amino acid residues 1-40) and the ligand binding domain (amino acids residues 228-526) were less conserved among mammalian ERβ

FIG. 3-2. Alignment of the deduced amino acid sequence of porcine embryo ERβ with those of other mammalian ERβ protein sequences. Only those amino acids that differ from porcine embryo ERβ are shown for bovine, ovine, human, rat and mouse sequences. Amino acids that are either missing or not reported for a particular species are indicated by (-).

```
PORCINE                                                                                                  MSICTSSHKEFSQLEPSED  19
BOVINE                                                                                                   ....................
OVINE                                                                                                    ....................
MOUSE                                                                                                    ....................
RAT                                                                                                      ....................
HUMAN                                                                                                    ....................

PORCINE    1  MDVKNSPSNLHSPVSYMCSQSVLPLEPGPIYIPSSYVESCHEYSAHTFYSPAVVNYSI-  58
BOVINE     1  .......S.........G..I.........L.....R.....V.....M.....-  58
OVINE      1  .......S.........G..I.........L.....R.....V.....M.....-  58
MOUSE      1  .EI....S.T..A.....I....M.........R.............H...V-  58
RAT       20  .EI....S.S..A.....I....M.........DWR...........M..UPG  79
HUMAN      1  ..I....S....S.....I....M.S.......D.M...P.......M.....-  58

PORCINE   59  -SS---MS---HVGPGRQATSPWVLWPTPGHLSPLAIHCQPSLLYAEPQKSPWCDTRSLE  111
BOVINE    59  -PW----.----D....T.....................V.........RE.....  111
OVINE     59  -PW----.----D....T.....................S.........R.....  111
MOUSE     59  -P.STG.L---G..V..TA.....S.....T...S.........EA....  114
RAT       80  ST.---.L---DG..V..S.....S.....T...S.........EA....  133
HUMAN     59  -P.---.VTNL.G....T.....................VV.R.L.N.....EA....  114

PORCINE  112  MTLPVWKETLKKASGSSCASPVTSPSSKEDAHCCAVCSDYASGYHYGVWSCEGCKAFFKK  171
BOVINE   112  .....................A.....................................  171
OVINE    112  ..F..................S.....................................  171
MOUSE    115  ............LG..G.......A.....................................  174
RAT      134  ............L.......NA.....................P...............  193
HUMAN    115  ............V..NR.....G.G.....................................  174

PORCINE  172  RSIQGHNDYICPATNQCTIDKNRKKSCQACRLRKCYEVGMVKCGSRKERCGKRVVKCQEN  231
BOVINE   172  .....................................................R...  231
OVINE    172  .....................................................R...  231
MOUSE    175  .....................................................R..S  234
RAT      194  .....................................................R..S  253
HUMAN    175  .....................................................L..R..S  234

PORCINE  232  SEGHLNCLSPAKGGWDHTTRWKELLLSTLSPEQLVLTLLEAEPPHVLVSRPSTPETEASM  291
BOVINE   232  .DEQ....KT.R..GPH.........A.....................I.........  291
OVINE    232  .DEQ....KT.R..APH.........A.....................M...A.....  291
MOUSE    235  ASEQV...NK..RTSG..P.....NS.....................W.....M.....  294
RAT      254  .SEQV...K..R..GAP.....................................M.....  313
HUMAN    235  ADEQ...AGK..RS.GAP..R...DA.....................I....A.....  284

PORCINE  292  MMSLTKLADKELVHMISWAKKIPGFMELSLYDQVELLESCWLEVLMVGLMWRSIDHPGKL  351
BOVINE   292  .....................V.....................................  351
OVINE    292  .....................V.....................................  351
MOUSE    295  ..............G.....V...L.....M.....................-----  354
RAT      314  ..............G.....V...L.....M.....................-----  373
HUMAN    295  .....................V...F.....M...M.....................  354

PORCINE  352  IFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILLNSSMYP-S  410
BOVINE   352  ......I..............................................-.  410
OVINE    352  .....................................................-.  410
MOUSE    355  .....................G.....A.....................------L  413
RAT      374  .....................................................-L  432
HUMAN    355  .....................................................LV  414

PORCINE  411  A-RA-QEAESSRELTHLLNAVTDALVWVIARSGISSQQQSVELAHLLHCLSSVRHASNKG  468
BOVINE   411  ..T.P...D.G.....................K..H.....H.....................  469
OVINE    411  .T.S-...D.G.....................K..H.....H.....................  469
MOUSE    414  .T.S-.....................SK.....................I.....  472
RAT      433  .-S.N.....................K.....................I.....  491
HUMAN    415  T-.T-.D.D....A.....................K.....H.....................  472

PORCINE  469  TEHLLNMKCKNVVPVYDLLLEMLNAHVLRGVKSLVTGSEESQMEEESESKEGSQKPQAQ  526
BOVINE   470  M.....................................MLVD.....  527
OVINE    470  M.....................S..P...R...MLRD.....  527
MOUSE    473  M...S.....................V..SIS...CGST.D.K.....NL.S.  530
RAT      492  M...S.....................V.PSIS...CSTSD.KN..S..NL.S.  549
HUMAN    473  M.....................V...C..SI....C.PA.D.K.....N..S.  530
```

proteins. By contrast, the region encompassing amino acid residues 141 to 227, which spans the DNA binding domain of this nuclear receptor, exhibited 100% identity among these species.

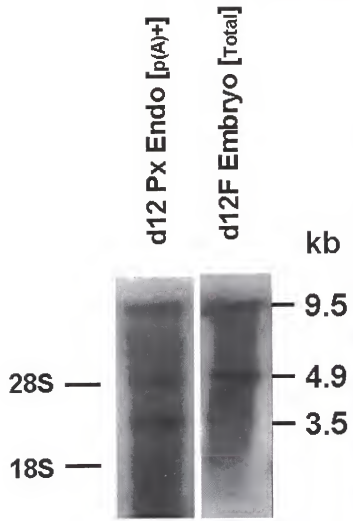
Characterization of Porcine ERβ mRNA and Protein

Total RNA prepared from F embryos collected at d12 of pregnancy and poly(A)$^+$ RNA isolated from d12 pregnant pig endometrium were used in Northern blot analysis, using a radiolabeled probe corresponding to the N-terminal region (nucleotides 1 to 491) of porcine embryo ERβ mRNA. As shown in Fig. 3-3, multiple transcripts ranging from 9.5 to 3.5 kb were observed in d12F embryos, in agreement with the sizes described for ERβ mRNA in other species (Tremblay et al., 1997). Pregnant porcine endometrium displayed these same multiple transcripts (Fig. 3-3), although these were barely detectable when total tissue RNA, instead of poly(A)+ mRNA was used (data not shown). Interestingly, whereas the 4.9 kb transcript was predominant in d12F embryos, the expression of the three major transcripts were comparable in the corresponding endometrium.

Immunolocalization of ERβ

Immunohistochemical analyses showed that ERβ protein was localized within the nuclear compartment of trophectoderm cells in d12F porcine embryos (Fig 3-4B, D). Positive staining for ERβ was also shown in uterine tissue sections prepared from a d60 pregnant pig (Fig 3-4F) . In the latter, immunostaining was

FIG. 3-3. Northern Blot analysis of porcine tissues for ERβ expression. Total RNA isolated from day 12 filamentous (d12F) embryos (30 μg) and poly (A+) RNA (5 μg) prepared from d12 pregnant pig endometrium (d12 endo) total RNA were hybridized to a porcine ERβ cDNA fragment. The transcript sizes are indicated on the right side of the panel.


d12 Px Endo [p(A)+]
d12F Embryo [Total]
kb
9.5
4.9
3.5
28S
18S

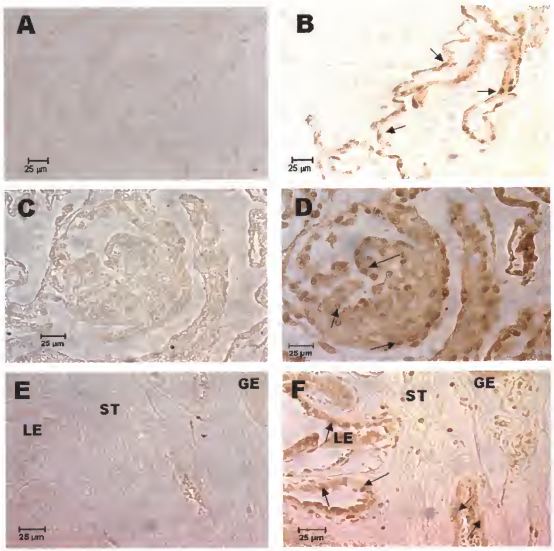
restricted to luminal and glandular epithelial cells and was not detected in stromal cells. Parallel serial sections of day 12F embryo and d60 pregnant uterine tissues did not show any staining, when incubated with normal rabbit IgG at concentrations equal to that of anti-rat ERβ antibody (10 μg/ml) (Fig 3-4A, C, E).

The presence of embryonic ERβ can be correlated with growth status, since immunolocalization of the growth-associated marker protein PCNA was shown to be present in the nucleus of d12F porcine embryo trophectoderm cells (Vale-Cruz, 2001).

Expression by Peri-implantation Embryos of ERβ and ERα

Primer sets for the detection of porcine ERα and ERβ transcripts in peri-implantation embryos were synthesized (Table 3-2), and the products from each PCR reaction were confirmed for authenticity by nucleotide sequence analysis (data not shown). Primers sets were also designed to detect transcripts for PR, cytochrome P450 aromatase Type III, cyclin D1, and GAPDH, in the same tissues. Porcine embryos collected at different stages of pregnancy (d11, 12, 13, and 14), were separated according to size and morphology (spherical S; tubular T; filamentous F), and analyzed for expression of the specific mRNAs by semi-quantitative RT-PCR. To ensure that the amounts of the starting material for the PCR reactions were the same for all samples, the yields from the RT reactions were equalized using the housekeeping gene encoding GAPDH (Fig. 3-5). In this strategy, the amount of cDNA that would yield an equivalent amount of GAPDH PCR product was determined for each cDNA preparation, and this

FIG. 3-4. Immunolocalization of ERβ protein in porcine embryos (day 12 filamentous) and porcine uterine endometrium (pregnancy day 60). Embryo (A-D) and uterine endometrial (E, F) tissue sections were incubated with either normal rabbit IgG (A, C, E) or polyclonal anti-rat ERβ IgG (B, D, F) at equal concentrations. Panels A/B and C/D represent distinct embryos photographed at different magnifications. Arrows depict representative nuclear staining for immunoreactive ERβ. GE, glandular epithelial; LE, luminal epithelial; ST stroma.


A
25 µm
B
25 µm
C
25 µm
D
25 µm
E
GE
ST
LE
25 µm
F
ST
GE
LE
25 µm

aliquot was then used in all PCR reactions for each cDNA sample.

As shown in Fig. 3-5, the levels of ERβ mRNA varied with embryo morphology. ERβ gene expression was low in S embryos (5-9 mm), increased progressively in embryos at the T stages, and reached maximal levels in F embryos at d12 of pregnancy. A decline in ERβ gene expression was subsequently observed in F embryos at d14 of pregnancy (Fig. 3-6). By contrast, ERα gene expression was barely detectable (and only after Southern blot hybridization analysis) and remained constant for all embryos of different morphologies (Fig 3-5A). PR mRNA expression was low in S embryos, increased in T embryos, and was significantly diminished in F embryos (Fig 3-5B, 6). The expression of cyclin D1, a gene positively correlated with growth was low at S stages, increased significantly by the T stage, and reached maximal levels in d12F, with a significant decline observed in d14F (Fig 3-5A,6). The pattern of P450 aromatase Type III gene expression was consistent with results of a previous study from this laboratory (Green et al., 1995), with expression gradually increasing from small (5-6 mm) to large (8 to 10 mm) S embryos, reaching peak levels for T embryos, and moderately decreasing in d12F embryos to nearly undetectable levels by d14 (Fig. 3-5A).

Embryo Gene Expression in Response to E_2 Treatment In vitro

To evaluate if the ERβ gene expressed in porcine d12F embryos is functional, experiments were conducted to analyze the effects of added 17β estradiol (E_2) on these embryos' pattern of gene expression, by RT-PCR.

FIG. 3-5. Developmental expression patterns of porcine embryonic genes. Relative expression levels of ERβ, Cyclin D1, P450 Aromatase (Type III), PR, and ERα genes were determined by RT-PCR, using RNAs isolated from embryos of different morphologies (S, spherical; T, tubular; F, filamentous) or sizes (5-9 mm diameter for S embryos). Each morphological stage is represented by 4 to 5 embryos from different pigs at the indicated pregnancy days. A total of 32 independent litters on days 11, 12, 13, and 14 of pregnancy were used. Two different day 30 pregnant pigs were used as source of placenta. Panels A and B represent RT-PCR reactions carried out using RNA samples from different embryos. A) Photographs of ethidium bromide-stained gels (for ERβ, Cyclin D1, and GAPDH) or autoradiograms (for PR and ERα), where each lane for each panel was loaded with 20 µl of the resultant PCR reaction, B) Photographs of ethidium bromide-stained gels for P450 aromatase (Type III) and GAPDH transcripts. The sizes of the PCR fragments are indicated at the right side of each panel.

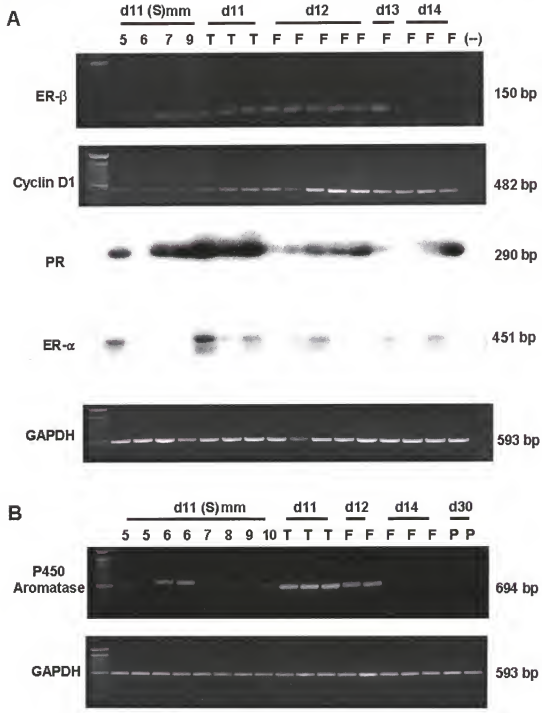
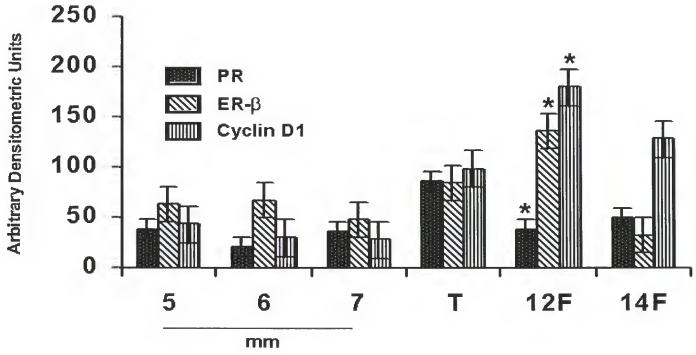

A
d11 (S)mm
d11
d12
d13
d14
5 6 7 9 T T T F F F F F F F F (–)
ER-β
150 bp
Cyclin D1
482 bp
PR
290 bp
ER-α
451 bp
GAPDH
593 bp
B
d11 (S)mm
d11
d12
d14
d30
5 5 6 6 7 8 9 10 T T T F F F F F P P
P450 Aromatase
694 bp
GAPDH
593 bp

FIG. 3-6. Diagrammatic representation of the expression levels for ERβ, Cyclin D1, and PR genes, after densitometric analysis of the PCR bands generated for each sample (panels A and B). Densitometric values were normalized to that for GAPDH and are presented as LSM ± SEM. Asterisk for the indicated developmental stage represents expression levels that differ significantly ($P < 0.05$) from all other examined time points for each gene.


250
200
150
100
50
0
Arbitrary Densitometric Units
PR
ER-β
Cyclin D1
*
*
*
5
6
7
T
12F
14F
mm
EMBRYO MORPHOLOGY

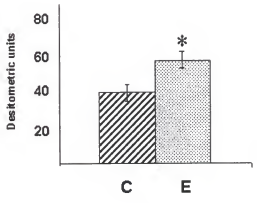
Embryos exposed to E_2 had increased levels of ERβ transcripts over those of control embryos receiving only vehicle (Fig 3-7A). This increase in ERβ levels was accompanied by an increase in P450 aromatase gene expression (Fig. 3-7B), which was normally undetectable in d12F embryos in explant culture. The expression of PCNA was evaluated as an indicator of cell proliferation. A decrease in PCNA gene expression was observed in embryos treated with E_2 compared with control embryos (Fig 3-7C). However, no changes were observed on cyclin D1 gene expression in embryos treated with E_2 compared with control embryos (Fig 3-7D). In these studies, gene expression levels were normalized to that of GAPDH.

Discussion

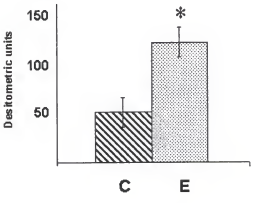
The peri-implantation period in porcine gestation is characterized by the production of copious amounts of estrogenic compounds by the developing embryo (Gadsby et al., 1980; Pusateri et al., 1990). These estrogens are known to stimulate uterine secretions (Roberts et al., 1993). However, the potential effect of estrogens on the embryo itself remains unknown. The present study reports the complete coding sequence for the porcine embryo ERβ protein. Analysis of the DNA sequence revealed an open reading frame of 1581 nucletotides which encoded a 526 amino acid protein. Comparison of the porcine ERβ nucleotide sequence with those of ovine and bovine (GeneBank accession no. AF110402 and no. AF177936 respectively) showed a 92% homology with a 3

FIG 3-7. Effect of 17β estradiol on porcine day 12 filamentous embryo gene expression in vitro. Five independent litters of filamentous embryos were incubated in culture medium (M199) in the presence (E) or absence (C) of added 17β estradiol (50 nM) for 24 h. Four μg of each RNA preparation was subjected to RT-PCR with specific primers for ERβ, P450 aromatase (Type III), PCNA, and Cyclin D1. Twenty μl of each PCR reaction was resolved in an agarose gel and densitometric values for each band, normalized to that of corresponding GAPDH were used for statistical analysis. Asterisk above each bar represents significant difference ($P < 0.01$) from control values.

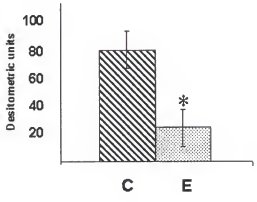
ER-β



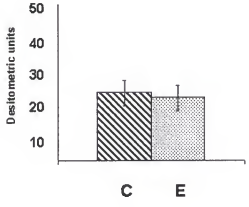
P450 aromatase



PCNA



Cyclin D1



nucleotide gap on the porcine sequence at position 1242. The deduced porcine ERβ amino acid sequence showed 90% homology to the bovine and ovine proteins, where a proline from porcine ERβ is missing at position 414. During the process of cloning ERβ cDNAs, several primer sequences were designed based on the conserved regions among human, mouse and rat (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The result of the cloning process was the isolation of a transcript which correspond to the longest isoform of ERβ previously reported in other species (Moore et al., 1998). The possibility of the presence of other isoforms in the porcine embryo remain open, since the primer sets used amplified preferentially the longest isoform whereas Northern Blot analysis demonstrated the presence of multiples transcripts consistent with multiple isoforms.

Immunohistochemistry on day 12 filamentous embryo sections using an anti-rat ERβ antibody showed porcine ERβ protein to be localized to the nucleus of the trophectoderm cells. Although previous studies have localized ERα, and to a more limited extent ERβ, in embryos of different species (Hou et al., 1996; Ying et al., 2000), a direct comparison of the expression levels of these steroid receptors as a function of peri-implantation development has not been conducted. Moreover, the functional implication of the expression of these receptors by the developing embryos has not been evaluated. The data presented here demonstrate that expression of ERβ is significantly higher and more tightly regulated than that of ERα in developing porcine embryos and the pattern of expression for ERβ mRNA parallels that of cyclin D1 during embryo

development, suggesting a functional linkage between ERβ and embryonic growth.

The rapid change in embryo morphology from tubular to filamentous coincident with maximum estrogen content within the uterine lumen (Gadsby et al., 1980), suggests estrogen action in embryo development. Analysis of cyclin D1, a gene positively correlated with cell growth, showed an increase in its expression by the time of embryo elongation. Maximum concentration of estrogen at the time of elongation and the action of estrogen, possibly occurring through ERβ to regulate PCNA expression, further suggest a possible role of ERβ on the control of embryo growth. It is worth noting that maximum concentrations of IGF-I in the uterine lumen occur at the same time as that of estrogen (Ko et al., 1994). Also, porcine embryos express IGF receptors (Green et al., 1995). Taken together these results suggest a stimulation of embryo growth by IGF-I and the potential regulation of that rapid growth by ERβ. Moreover, IGF-I is known to phosphorylate ERβ and to potentiate its action in stimulating gene transcription (Tremblay et al., 1999). The enhancement of ERβ activity by phosphorylation could mediate the increase in P450 aromatase gene expression during the embryo elongation process, since in vitro studies showed stimulation of P450 aromatase gene expression in embryos treated with E_2, thus further transcriptional activity of ERβ could be reached by phosphorylation.

The differential expression of ERβ dependent on stage of embryo development provides a mechanism whereby asynchrony in embryo growth could result in increased embryo mortality. These rapidly growing embryos at day

12 express high levels of ERβ and that increase in ERβ expression parallels embryo maximal growth. The latter correlates with previous reports demonstrating that in rapid growing tissue, ERβ gene expression is increased, suggesting that its function is to slow down growth (Iafrati et al., 1997; Lindner et al., 1998). Also recent studies have shown a physical interaction of the ERβ protein with proteins involved in the regulation of the cell cycle such as MAD2, a molecule that serves as a check point during the process of mitosis allowing cells to divide when chromosomes are perfectly aligned (Poelzl et al., 2000). Since the predominant estrogen receptor present in the embryo is ERβ and the in vitro culture data showed a reduction in PCNA expression with E, our data suggests that one of the roles for this receptor is to slow growth of the embryo. However since these embryos do not grow in synchrony, the effect of estrogen in embryos less developed by the time of maternal recognition of pregnancy could further slow embryo growth and impede development. As a further evidence of this relationship, a breed of pigs known for its low embryonic mortality (Meishan) is characterized by lesser asynchrony in embryo development (Ford, 1997) and lower embryonic production of estrogens (Wilson and Ford , 1997).

The pattern of expression of ERβ mRNA during embryo development which reaches a maximum expression at the time of the highest levels of estrogen content in the uterine lumen (Fig. 3-6) and the ability of estrogen to increase the levels of ERβ mRNA in vitro, suggest the presence of cis acting elements in ERβ genes that can mediate E-stimulation of ERβ transcription. Indeed, sequence analysis of the human ERβ promoter region demonstrated the

presence of an estrogen response element (Li et al., 2000).

The regulation of P450 aromatase gene expression in the porcine embryos is not well understood. This gene is very tightly regulated around peri-implantation. P450 aromatase gene expression is induced in large S embryos, reaching peak levels by the tubular stage (d11-d12), with a decrease in filamentous embryos (d12), to undetectable levels by day 14 (Green et al., 1995). This transient expression of P450 aromatase follows the pattern of estrogen concentrations in the uterine lumen (Pusateri et al., 1990). Here, we show that estrogen stimulates P450 aromatase gene expression. After 24 h of culture, filamentous embryos had undetectable expression of P450 aromatase gene, however, after treatment with estradiol 17β, the expression of this gene become detectable. These results suggest the regulation of P450 aromatase gene expression by estrogen acting through ERβ, since the latter is the predominant ER receptor in the embryo.

In a recent study, Kao et al. (2000) demonstrated that the major steroid product of the stably expressed porcine P450 aromatase blastocyst isoform mRNA is not estradiol, but rather 19-nortestosterone (N). The relevance of such observation in vivo is not clear at the present time, since measurements of N concentrations in uterine luminal fluids from peri-implantation uteri have not been carefully conducted. However, N has been shown to bind ERβ, albeit with low affinity (Kuiper et al., 1997); thus, if indeed high concentrations of N are present within the uterine microenvironment, these could compensate for its low affinity for ERβ and could conceivably influence ERβ-signaling pathways within the

embryo. The distinct functional consequences on embryonic growth and development of ERβ-bound to N from that bound to estradiol is an interesting question that requires further investigation.

Our results highlight a natural selection process possibly occurring within the uterus, whereby more developed embryos, through their estrogen production, inhibit the growth of less developed embryos expressing ERβ. The further elucidation of the potential involvement of ERβ on the mechanism underlying embryo mortality using the pig system, may provide clues to the etiology of implantation defects during early pregnancy in humans, whose embryos produce estrogens, albeit at lower levels than those of the porcine species (Edgar et al., 1993).

CHAPTER 4
IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ERβ IN PORCINE TROPHOBLASTIC CELLS

Introduction

In porcine gestation, the first 2 weeks after mating are crucial for embryo survival and the success of pregnancy. During this period of peri-attachment, embryos acquire the ability to produce and secrete copious amounts of estrogens (Gadsby et al., 1980), the signals for maternal recognition of pregnancy (Bazer et al., 1984). Between days 11 and 12, the embryos also undergo dramatic morphological changes from small spherical to large filamentous forms within a short period of time (Geisert et al., 1982). This periimplantation period is characterized by a high rate of embryo mortality (Pope and First, 1985). It has been pointed out that the high rate of embryo mortality is likely due to asynchrony in development among embryos (Ford, 1997). The synthesis and secretion of estrogens by more advanced embryos is believed to produce biological and functional changes in the uterine environment which are detrimental for the less advanced embryos (Roberts et al., 1993; Wilson and Ford, 1997). In the previous chapter (Chapter 3), we showed that embryos are potential targets of their own secreted estrogens since they express ER,

specifically ERβ. Previous research (Yelich et al., 1997) failed to detect expression of ERα in peri-implantation embryos, a finding consistent with our own (Chapter 3). ERβ gene expression was characterized during porcine embryo development (Chapter 3), and shown to occur coincident with that of estrogens in the uterine lumen (Gadsby et al., 1980; Pusateri et al., 1990). The peak of ERβ gene expression was also coincident with that of Cyclin D1, a known marker for cell proliferation. Further experiments using in vitro embryo cultures showed that embryonic ERβ was functional since exogenous estrogen decreased PCNA gene expression. Since the latter is a gene correlated with growth, this result suggested that estrogen is able to decrease growth through ERβ when the cells are proliferating rapidly. This is not surprising since several laboratories have now shown that in rapidly proliferating cells, the action of estrogen through ERβ causes a reduction in cell growth (Lindner et., 1998; Jarvinen et al., 2000). Moreover, ERβ has been shown to physically interact with a cell cycle regulatory protein MAD2, which is a check point on the mitosis process (Poelzl et al., 2000). In order to further dissect the possible pathways involved in E-ERβ action in porcine embryos, we characterized a newly developed cell line (pTr2) derived from trophectoderm cells of day 12 filamentous embryos (Ka et al., 2001). We report in this study, the presence of ERβ and PR but not ERα, mRNAs in the pTr2 cell line, and the localization of the ERβ protein in the nucleus of these cells. We further demonstrate the functionality of ERβ upon binding estrogen, using transcriptional assays of an ERE-reporter gene, and [^{3}H] thymidine incorporation assays into cellular DNA.

Materials and Methods

Materials

Optimizer PCR-Kit and cDNA cycle kit were purchased from Invitrogen (Carlsbad, CA), primers were obtained from Gemini, Taq polymerase was purchased from Boehringer Mannhein. TRIzol, Lipofectamine, Opti MEM-I, Hanks' Balanced Salt Solution (HBSS), and M199 were media purchased from Gibco BRL (Grand Island, NY). Fluorescein (FITC)-conjugated anti-rabbit and anti-rat antibodies, antibiotic-antimycotic (ABAM), fetal bovine serum (FBS), and Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM-F12 HAM) were purchased from Sigma Chemical Co (St Louis, MO.). [^{3}H] Thymidine (specific activity 6.7 Ci/mmol) was purchased from ICN Biotech (Irvine CA), and all molecular biology-grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Cultures

The porcine day 12 trophectoderm cell line (pTr2) was generously provided by Dr. Laurie Jaeger (Texas A&M) (Ka et al., 2001) and was grown in DMEM-F12 medium supplemented with 5% charcoal-stripped FBS, 0.1 U/ml insulin, and 1% ABAM. The porcine day 14 trophectoderm cell line (Jag-1, courtesy of J. Ramsoondar, Texas A&M University) were grown in M-199 medium without phenol red and supplemented with 10% FBS and 1% ABAM.

Medium was changed every other day. The cells were incubated at 37°C in an atmosphere of 95% air:5% CO_2.

RNA Isolation and RT-PCR Analysis of pTr2 and Jag-1 Cell Gene Expression

Total cellular RNA was isolated from pTr2 cells, JAG-1 cells, day 12 filamentous embryos and porcine ovary tissuefrom a day 12 pregnant pig with TRIzol according to the manufacturer's procedures. cDNAs were synthesized using equal amounts of total RNA (5 µg) prepared from these tissues or cells. The PCR amplification were performed in a total reaction volume of 50 µl containing 1 µl of cDNA template, 50 pmol of the forward and reverse primers (indicated in Table 2, Chapter 3), 1 mM each of dNTPs, 1X PCR buffer (Invitrogen) and 1U of Taq Polymerase. The number of cycles used were optimized as described in the previous chapter (Chapter 3). All PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide, and were visualized over UV light.

Immunofluorescence Localization of ERα and ERβ

The immunofluorescence detection of ERα and ERβ followed described protocols (Spector and Smith, 1986). In brief, cells at 50% confluence were rinsed in 10mM PBS (pH 7.4) and fixed in 4% paraformaldehyde. After fixation, cells were permeabilized with 0.2% Triton100 in PBS for 10 min at room temperature. Cells in different chambers were then incubated with either a rabbit polyclonal anti-rat ERβ antibody (previously described in Chapter 3) at a

concentration of 10 µg/ml or rat monoclonal anti-human ERα (H-222) antibody (courtesy of Dr. Geoff Greene, University of Chicago) at a concentration of 5 µg/ml for 1 h at RT. ERβ immunizing peptide corresponding to amino acids 54-71 of rat ERβ (YAEPQKSPWCEARSLEHT) (Upstate Biotechnology) at a concentration of 1 µg/ml was incubated with ERβ antibody for 1 h, and the mix was added to the cells, which was then incubated for another hour. Cells were washed 3 times with PBS/3%BSA, and incubated with fluorescein-conjugated anti-rabbit IgG (Sigma) for ERβ primary antibody (1:50 dilution) and anti-rat IgG(Sigma) for ERα antibody (1:50 dilution) for 1 h at room temperature. Slides were rinsed in PBS, dried and then overlaid with cover slips. Immunofluorescent signals were viewed using a Zeiss Axioplan 2 microscope equipped with RGB Spot Digital camera system with corresponding software (Carl Zeiss, Inc, New York, NY.).

Transfection and Luciferase (Luc) Assays

Transfections were performed on pTr2 cells, when 80%confluent, by the Lipofectamine method. Cells were transfected with 5 µg of $(ERE)_4$-tk-luciferase reporter plasmid (Chusacultanachai et al., 1999), 5 µg of pCMV5 vector and 5 µl of lipofectamine. In some experiments, cells were also co-transfected with 0.5 µg of hERα-pCMV5 expression vector per well. pCMV5 and hERα-pCMV5 were provided by Dr. Benita Katzenellenbogen (University of Illinois). Plasmid DNAs and lipofectamine were mixed with HBSS to a final volume of 200 µl and incubated for 0.5 h at RT. Cells were washed and then received 200 µl of the previous mixture plus 800 µl of Opti-media I and incubated for an additional 6 h.

After the incubation, 1 ml of DMEM-F12 containing 10% charcoal-stripped serum (CSS) was added per well and cells were incubated overnight at 37°C in an atmosphere of 5% CO_2, 95% air. Cells were washed with HBSS and medium was replaced with either serum-free (SF) or CSS-containing medium (5%) with added17β estradiol (50 nM) or medium-vehicle alone (ethanol). After 24 h cells were washed with PBS and lysed in single-strength lysis buffer (200 µl; Promega Corp., Madison, WI). The protein concentration of the cell lysate (5 µl) was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, CA). Quantitative determination of Luc activity (measured as relative light units) in cell lysate was carried out using an Autolumat Luminometer (EG&G Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to the protein concentration of cell lysates.

Thymidine Incorporation Assay

Cells were grown to confluence in 6-well culture dishes and then incubated in the appropriate serum-free medium for 24 h. Cells in monolayers were washed with HBSS, and replaced with either SF medium or CSS-containing medium (5%) and to which were added 17β estradiol (10, 50 and 500 nM). After 20 h of incubation at 37 °C in an atmosphere 5% CO_2, 95% air, 1 µCi of [^{3}H]thymidine per well was then added. After labeling for 4 h, cells were washed with PBS, fixed in methanol and precipitation was performed using two sequential incubations with trichloroacetic acid (TCA) at 5% for 10 min. Precipitate was recovered using 300 µl of NaOH (0.3 M). Radioactivity incorporated into the TCA-precipitable material was determined by liquid scintillation counting using a TRI-CARD Liquid Scintillation Analyzer (Packard,

Canberra, Australia).

Statistical Analysis

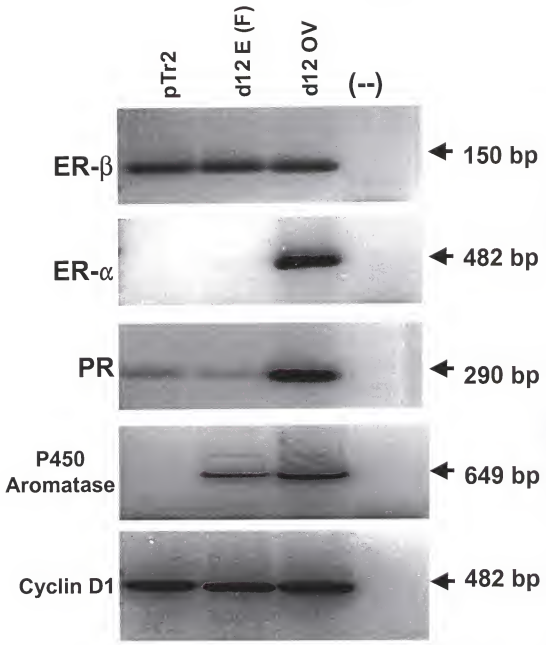
Data obtained from transfection and thymidine incorporation assays were subjected to least squares ANOVA using the General Linear Models procedures of SAS (Barr et al., 1979). For the transfection experiments, the model tested consisted of two variables: treatment and experiment plus the interaction of treatment by experiment. Both variables were considered fixed. For the co-transfection experiments, the model consisted of four factors: level of 17β estradiol, number of experiments, levels of CSS, levels of hERα expression vector, and all double, triple and quadruple interactions. All the variables were also considered fixed. For the thymidine incorporation assay, the model consisted of two variables: levels of 17β estradiol, number of experiments, and the interaction between the two. These variables were also considered to be fixed. Values were considered significant at $p<0.05$ and are presented as least squares means (LSM)$\pm$ SEM.

Results

Analysis of pTr2 and Jag-1 Cell Gene Expression

Primers previously used to amplify ERα, ERβ, and PR transcripts(Table 3-2) were similarly used to evaluate the expression of these steroid receptors in the pTr2 cells, by semi-quantitative RT-PCR assay. In these studies, porcine day 12 filamentous embryos and ovaries collected from a day 12 pregnant gilt

Fig. 4-1. RT-PCR analysis of ERβ, ERα, PR, P450 aromatase, and cyclin D1 mRNA from pTr2 cells, day 12 filamentous embryo [d12 E (F)] and maternal ovary from a day 12 pregnant gilt (d12 OV) mRNA. Each lane represents 20 ul of the resultant PCR reaction from each tissue. Reactions were separated in an agarose gel (1.5%) by electrophoresis, stained with ethidium bromide and visualized with UV light. Samples marked -- represent negative control in which cDNA was omitted from the PCR reaction. The sizes of the expected PCR products are shown in base pair (bp).


pTr2
d12 E (F)
d12 OV
(--)
ER-β
150 bp
ER-α
482 bp
PR
290 bp
P450
Aromatase
649 bp
Cyclin D1
482 bp

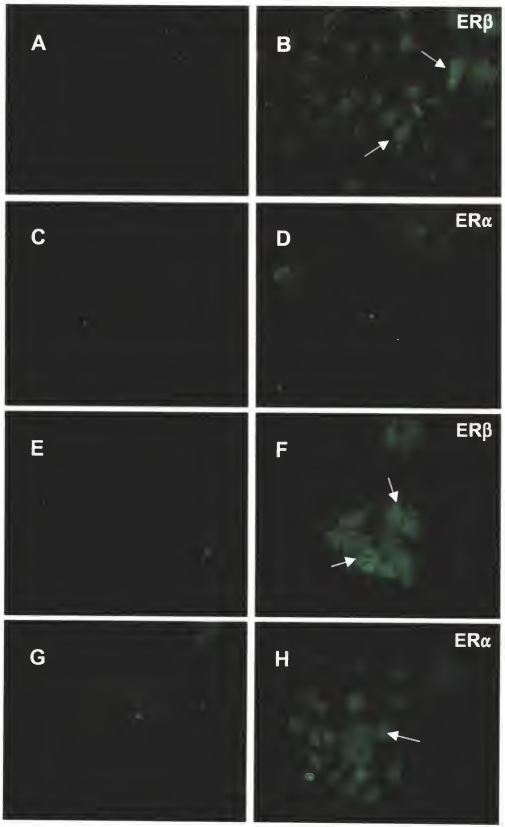
were used as positive controls. ERβ gene was expressed in all samples, and no major differences in the intensities of the ERβ transcripts were detected (Fig. 4-1). However, we could not detect ERα gene expression in pTr2 cells, consistent with our failure to also detect this ER isoform in d12F embryos (Chapter 3)(Fig. 4-1). The ERα primer sets, however, detected ERα transcripts in the porcine ovary. We were able to also detect PR gene expression in pTr2 cells as well as in d12F embryos and at much higher levels in the ovary (Fig. 4-1). We further characterized the expression of P450 Aromatase gene in pTr2 cells. Similar to our previous observations (Chapter 3) for d12F embryos in culture where after 24 h they had lost the capacity to express P450 aromatase, pTr2 cells also had no detectable aromatase gene expression (Fig. 4-1). By contrast, the expression of Cyclin D1 gene, a gene correlated with growth was detected in the cell line and whole embryos as well as in ovary (Fig. 4-1).

Immunolocalization of ERα and ERβ

The presence of the ERβ transcript in pTr2 cells was confirmed at the level of the protein by immunofluorescence using rabbit antibodies generated against rat ERβ . ERβ protein was detected in the nucleus of these cells (Fig. 4-2B), but not in those cells incubated with control rabbit IgG used at a concentration equal to that for anti-rat ERβ antibody (Fig. 4-2A). Ishikawa cells used as a positive control for ERβ detection, showed immunofluorescence signals for ERβ (Fig 4-2F), however, cells incubated in parallel with control rabbit IgG did not show any nuclear localized signal (Fig 4-2E).

Expression of ERα using specific anti-human rat monoclonal antibody for ERα were also examined in pTr2 cells and Ishikawa cells. ERα protein was not

Fig. 4-2. Immunolocalization of ERβ and ERα in pTr2 (B, D) and human endometrial carcinoma cells (Ishikawa) (F, H). pTr2 cells were incubated either with anti-rat ERβ rabbit polyclonal antibody (B) or normal rabbit IgG (A), and with anti-human ERα rat monoclonal antibody (D) or normal rat IgG (C). Ishikawa cells were incubated either with anti-rat ERβ rabbit polyclonal antibody (F) or normal rabbit IgG (E), and with anti-human ERα rat monoclonal antibody (H) or normal rat IgG (G). Signals were detected with indirect immunoflorescence using a fluorescein-conjugated goat anti-rabbit antibody and rabbit anti-rat antibody. Arrows denote nuclear staining.


A
B
ERβ
C
D
ERα
E
F
ERβ
G
H
ERα

detected in pTr2 cells (Fig. 4-2D), nor in those cells incubated with control rat IgG at a concentration equal to that used for anti-ERα antibody (5μg/ml) (Fig. 4-2C). However, Ishikawa cells used as a positive control in these studies clearly showed nuclear signals for ERα (Fig. 4-2H). Those cells incubated with control rat IgG did not show any nuclear staining for this protein (Fig. 4-2G).

In order to demonstrate the specificity of the anti-rat ERβ antibody, cells were incubated with ERβ antibody pre-incubated with an ERβ immunizing peptide (5μg/ml). When these cells were compared to those incubated with ERβ antibody alone (Fig. 4-3B), the nuclear signal obtained with the anti-ERβ antibody was diminished by the presence of the immunizing peptide (Fig. 4-3C). This result suggests that the nuclear signal generated by the anti-ERβ antibody used in these studies is specific to ERβ protein.

Functional Analysis of ERβ in pTr2 Cells

In order to verify that the ERβ present in pTr2 is functional, we performed a series of transfection experiments using an ERE-responsive Luc reporter gene construct. In the first experiment, cells were treated with E (50 nM) in the presence or absence of an antagonist to both ER isoforms namely, ICI-182780 (500 nM) which was kindly provided by ZENECA (Macclesfield, UK). A significant induction ($P<0.001$) in the transcriptional activity of the reporter gene construct was observed with added E. This activity was inhibited when ICI 182,780 was added with E (Fig. 4-4). These results suggest that ERβ present in these cells can mediate E-responsive transcriptional activity.

The effect of serum on basal promoter activity was also analyzed in transfection experiments (Fig. 4-5). Results indicate that serum significantly

Fig. 4-3. The specificity of anti-rat ERβ antibody was demonstrated by comparison of the intensities of immunoflorescence signals obtained in pTr2 cells incubated with either anti-rat ERβ antibody alone (B) or in the presence of immunizing peptide (IP) (C). Control cells were incubated with normal rabbit IgG (A). Signal was detected with indirect immunoflorescence using a fluorescein conjugated goat anti-rabbit antibody. Arrows denote nuclear staining.

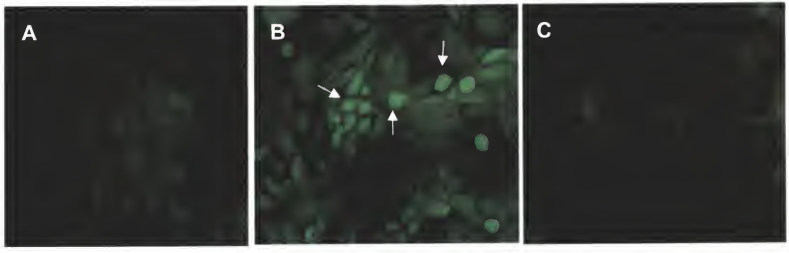
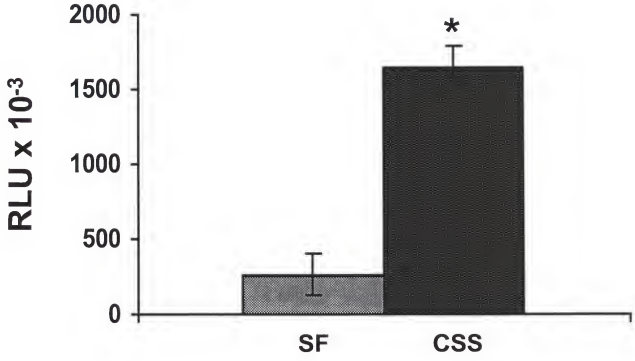

A
B
C
NRS IgG
Anti-ERβ
Anti-ERβ+IP

Fig. 4-4. Transient transfection of pTr2 cells with 4XERE-tk-LUC reporter gene. Cells were transfected with 5 µg of the reporter gene and treated with E or E+ICI 182,780, or vehicle (V) alone. Treatments were done under serum-free (SF) conditions. Promoter activity was measured as Relative Luciferase Units (RLU). Each group represents 3 independent experiments of 3 replicates each. Results are presented as LSM± SEM. Significance at $P<0.001$ is denoted by * .

RLU x 10-3
10000
8000
6000
4000
2000
0
*
V
E
E+ICI

Fig. 4-5. Transient transfection of pTr2 cells with 4XERE-tk-LUC reporter gene. Cells were transfected with 5 µg of the reporter gene and placed in serum-free (SF) or charcoal-stripped serum (CSS) containing medium. Promoter activity was measured as Relative Luciferase Units (RLU). Each group represents 3 independent experiments of 3 replicates each. Results are presented as LSM$\pm$ SEM. Significance at $P<0.001$ is denoted by * .


2000
1500
1000
500
0
RLU x 10-3
*
SF
CSS

($P<0.001$) increased the activity of the reporter gene promoter construct compared to that under SF conditions (Fig. 4-5). These data suggest an E-independent promoter transactivation by serum-derived factors.

Using the same ERE-responsive Luc reporter gene construct, a series of co-transfection assays with a human ERα expression vector were performed to determine if functional interactions exist between pTr2 cell ERβ and the transfected hERα. In the first experiment, treatments were done under SF conditions. Cells treated with E had a significant ($P<0.001$) 4-fold increase in luciferase activity compared to control cells (Fig. 4-6A). Cells that received hERα expression construct and E had a significant ($P<0.001$) 2-fold increase in luciferase activity compared to those cells that just received the hERα expression construct (Fig. 4-6A). Comparison between cells that received hERα with cells that received the empty vector (pCMV5) showed a significant increase ($P<0.001$) in the basal transcription activity of the reporter construct (Fig. 4-6A). The second set of experiments followed the previous study but was carried out in CSS. Cells treated with E have a significant ($P<0.001$) 2.2-fold increase in luciferase activity over control cells (Fig. 4-6B). Cells that received the hERα expression construct and E had a significant ($P<0.001$) 1.9-fold increase in luciferase activity over cells that received the hERα expression construct only (Fig. 4-6B). Under conditions of CSS-containing medium, cells that received the hERα expression compared to those cells that just received the empty vector (pCMV5) had a significant ($P<0.001$) decrease in the basal transcription activity of the reporter construct compared to the transcription activity observed when the studies were carried out under SF conditions (Fig. 4-6B).

We analyzed the effect of transfected hERα on the transcriptional activity of the E-responsive reporter gene construct in pTr2 cells as a function of CSS.

Fig. 4-6. Co-transient transfection of pTr2 cells with 4XERE-tk-LUC reporter gene and hERα expression vector (pCMV5-hERα). Cells were transfected with 5 µg of the reporter gene along with 0.5 µg of either the hERα expression vector or the pCMV5 empty vector in the presence or absence of added E. Treatments were done in either A) serum-free (SF) or B) charcoal-stripped serum (CSS) containing medium. Promoter activity was measured as Relative Luciferase Units (RLU). Each group represents 3 independent experiments of 3 replicates each. Results are presented as LSM$\pm$ SEM. Significance differences are denoted by different letters ($P<0.001$).

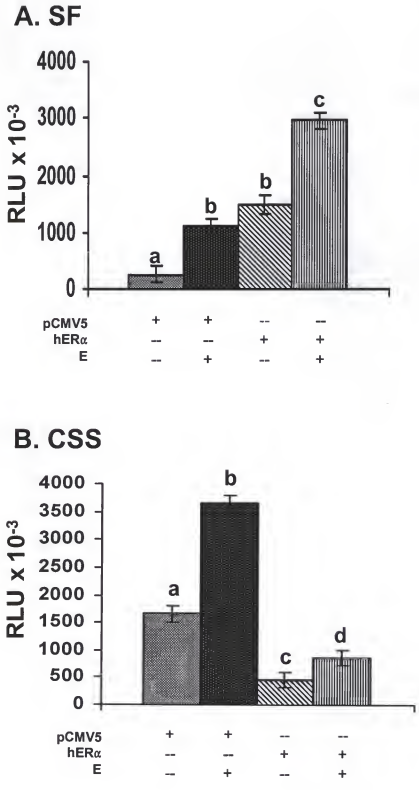
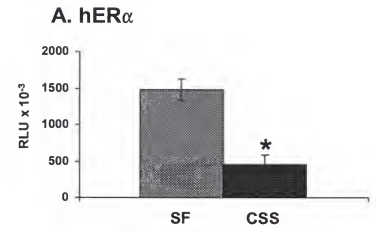
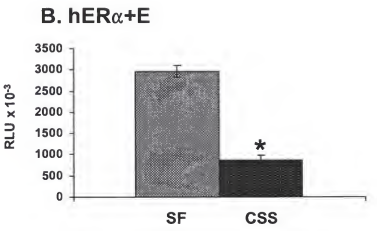
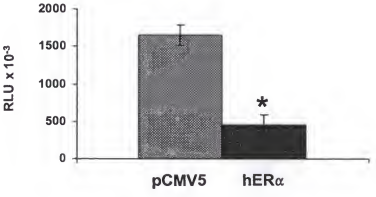
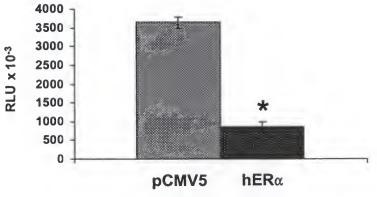

A. SF
RLU x 10-3
4000
3000
2000
1000
0
a
b
b
c
pCMV5 + + -- --
hERα -- -- + +
E -- + -- +
B. CSS
RLU x 10-3
4000
3500
3000
2500
2000
1500
1000
500
0
a
b
c
d
pCMV5 + + -- --
hERα -- -- + +
E -- + -- +

Fig. 4-7. Analysis of cotransfection experiments using pTr2 cells with 4XERE-tk-LUC reporter gene and either hERα expression vector or pCMV5 empty vector. A) Comparison of pTr2 cells cotransfected with 4XERE-tk-LUC reporter gene and hERα reporter gene under SF or CSS conditions. B) Comparison of pTr2 cells cotransfected with 4XERE-tk-LUC reporter gene and hERα reporter gene and supplemented with E under SF or CSS conditions. C) Comparison of pTr2 cells cotransfected with 4XERE-tk-LUC reporter gene and either hERα reporter gene or pCMV5 empty vector under CSS conditions. D) Comparison of pTr2 cells cotransfected with 4XERE-tk-LUC reporter gene and either hERα reporter gene or pCMV5 empty vector supplemented with E under CSS conditions. Luciferase activity was measured as a relative luciferase units (RLU). Each group represent 3 independent experiments of 3 replicates each. Results are represented by LSM$\pm$ SEM. Significance is denoted by * and $P<0.001$.


A. hERα
RLU x 10-3
2000
1500
1000
500
0
*
SF
CSS


B. hERα+E
RLU x 10-3
3500
3000
2500
2000
1500
1000
500
0
*
SF
CSS


C. CSS
RLU x 10-3
2000
1500
1000
500
0
*
pCMV5
hERα

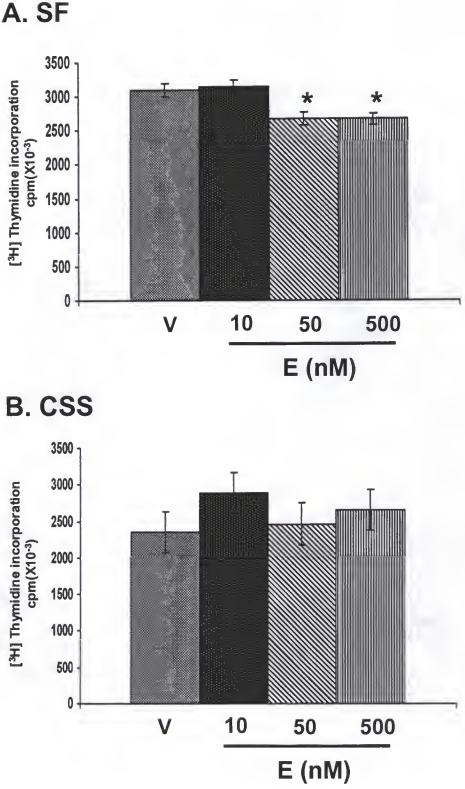

D. E+CSS
RLU x 10-3
4000
3500
3000
2500
2000
1500
1000
500
0
*
pCMV5
hERα

pTr2 cells transfected with hERα expression construct and under CSS condition showed a significant ($P<0.001$) 3.2-fold decrease in promoter activity compared to those cells with transfected hERα expression, but under SF conditions (Fig. 4-7A). In the same manner, pTr2 cells that received hERα expression vector, E, and under CSS conditions had a significant ($P<0.001$) 3.4-fold decrease in luciferase activity compared to those cells that received the same treatments, but under SF conditions (Fig. 4-7B). To further characterize this negative effect, cells in CSS were transfected with either hERα or the empty vector (pCMV5). Those cells that received hERα expression vector had a significant ($P<0.001$) 3.6-fold decrease in luciferase activity compared with those cells that just received the empty vector (pCMV5), and this decrease was independent of estrogen ligand (Fig. 4-7C). Finally, cells under the CCS condition with E were transfected either with hERα expression construct or the empty vector. Those cells that received the hERα expression construct had a significant ($P<0.001$) 4.2-fold decrease in luciferase activity compared to cells that received the empty vector (pCMV5) (Fig. 4-7D). These results suggest a negative interaction between serum factors and hERα in the regulation of the ERE-expression construct activity independent of E.

Effect of Estrogen on pTr2 Cell Proliferation

We have previously shown that exogenous E treatment of d12F embryos for 24 hr resulted in decreased PCNA gene expression, PCNA being considered a positive marker of growth (Chapter 3). These results suggest that ERβ mediates E-inhibition of cell growth/proliferation. To determine whether pTR2 cells respond similarly to E, the effect of E on DNA synthesis in pTr2 cells was

Fig. 4-8. Effect of different levels of 17β estradiol (E) or vehicle (V) alone (100% EtOH) on DNA synthesis of pTr2 cell line as measured by [^{3}H] thymidine incorporation under either A) serum-free (SF) or B) charcoal-stripped serum (CSS) containing medium. Each group represents 3 independent experiments of 3 replicates each. Results are represented as LSM$\pm$ SEM. Significance at $P<0.05$ is denoted by *.


A. SF
[³H] Thymidine incorporation cpm(X10⁻³)
3500
3000
2500
2000
1500
1000
500
0
*
*
V
10
50
500
E (nM)
B. CSS
[³H] Thymidine incorporation cpm(X10⁻³)
3500
3000
2500
2000
1500
1000
500
0
V
10
50
500
E (nM)

examined by incorporation of tritiated thymidine into cellular DNA when grown under different concentrations of 17β estradiol (0, 50 and 500 nM, in SF and CSS conditions). Under SF conditions, there is a small but significant reduction ($P<0.05$) in DNA synthesis when E concentration is 50 and 500 nM, respectively(Fig. 4-8A). Under charcoal-stripped serum conditions, no effect on DNA synthesis was observed with all E concentrations tested (Fig 4-8B).

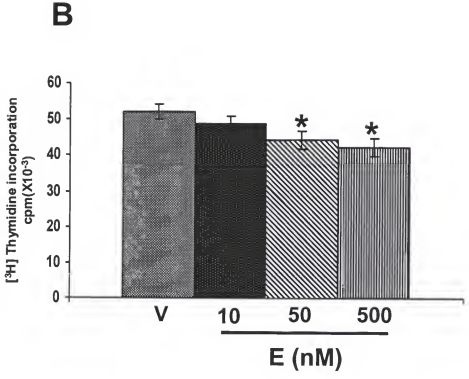
We performed the same experiments using a d14 trophectoderm-derived cell line JAG-1. This cell line exhibited expression of ERβ gene but not that of ERα, similar to that shown for d14F embryos from which this cell line was derived (Fig 4-9A) . A reduction by E on DNA synthesis in these cells was observed, consistent with the inhibitory effect of E, via ERβ on DNA synthesis in pTr2 cells (Fig. 4-9B).

Discussion

Estrogens produced and secreted during the time of periimplantation by the porcine embryos are crucial for sustaining pregnancy in the pig (Bazer et al., 1982). It is during this time when 30 to 40 % of embryonic loss occurs (Pope and First, 1985). Estrogen has been implicated to mediate embryonic death (Ford, 1997). We previously showed that E originating mainly from embryos, can target not just the uterus but also the embryo itself through the binding of ERβ (Chapter 3). In those experiments, embryos cultured in vitro in the presence of E exhibited regulated expression of genes associated with the growth process. In the present study, we characterized the expression of ER and PR in the pTr2 cell line. pTr2 cells were derived from dispersed cells of day 12 filamentous embryos

Fig. 4-9. Characterization of ERβ expressed by Day 14 trophectoderm cell line Jag-1. A) RT-PCR analysis for ERβ on Jag-1 cells and day 14F embryo mRNA. Each lane represents 20 ul of the resultant PCR reaction from each sample. Reactions were separated in an agarose gel (1.5%) by electrophoresis, and PCR fragments were stained with ethidium bromide and visualized with UV light. The size of the expected PCR product is shown in bp. B) Effect of different levels of 17β estradiol (E) or vehicle alone (V) (100% EtOH) on DNA synthesis of Jag-1 cell line as measured by [^{3}H] thymidine incorporation in serum-free (SF) containing medium. Each group represents 3 independent experiments of 3 replicates each. Results are represented by LSM$\pm$ SEM. Significance at $P<0.05$ is denoted by *.

A
JAG-1
D14 E (F)
ER-β
150 bp


B
[³H] Thymidine incorporation cpm(X10⁻³)
60
50
40
30
20
10
0
*
*
V
10
50
500
E (nM)

(Ka et al., 2001). These cells, similar to day 12F embryos express ERβ mRNA transcripts as detected by RT-PCR. We also demonstrated the presence of ERβ protein in these cells, but could not detect the presence of ERα. In these studies, we used a human endometrial carcinoma cell line (Ishikawa) to serve as a positive control (Nishida et al., 1985) since this cell line have been reported to express PR and ERα (Hata and Kuromoto, 1992). We found that the lack of signal using the ERα antibody in pTr2 cells was specific, since the antibody recognized ERα protein in Ishikawa cells.

To further demonstrate the specificity of the ERβ protein signal obtained from the immunofluorescence studies, we used an oligopeptide corresponding to a part of the rat ERβ protein sequence. Pre-incubation of the oligopeptide with the antibody prevented the detection of the nuclear signal generated by the anti-ERβ antibody alone, suggesting the authenticity of the immunofluorescence signal obtained with the ERβ antibody.

PR transcript(s) was also detected in pTr2 cells by RT-PCR, and the levels of its expression were comparable to those found in day 12F embryos. The significance of PR expression by developing embryos is still unknown. However progesterone has been reported to be present in uterine luminal fluids (Geisert et al., 1990), suggesting a possible role for progesterone in regulating uterine as well as embryo functions.

P450 aromatase, the enzyme required for the aromatization process in the conversion of androgen to estrogen (Thompson et al., 1974) is expressed by the porcine embryo in a very transient and tightly regulated fashion during the time of periimplantation (Green et al., 1995). The regulatory mechanism behind the transient expression of P450 aromatase gene is still unknown, however

some factors have been associated with the regulation of the P450 aromatase gene in porcine embryos including IGF-I (Green et al., 1995) and estrogen (Chapter 3). One important observation in the behavior of pTr2 cells is that they do not express P450 aromatase; this agrees with our previous observation (Chapter 3) where day 12F embryos placed in culture for 24 hours lose expression of P450 aromatase gene. This loss in expression of P450 aromatase gene in culture suggests a very complicated interaction that occurs between embryos and compounds present in the uterine environment such as retinol. The expression of retinoic acid receptors by the porcine embryos has been previously reported (Yelich et al., 1997), and large amounts of retinol are found in the luminal fluids (Roberts et al., 1993). Since recent reports in a human choriocarcinoma cell line (JEG3) and breast cancer cells (MCF-7) have shown the effect of retinoic receptor in increasing P450 aromatase gene expression (Sun et al., 1998; Mu et al., 2000), retinol could be one of the many factors involved in the regulation of P450 aromatase expression in porcine embryos; however, we have no indication that this factor is present in our in vitro culture system. This could explain why d12F embryos in culture for 24 h lost the ability to express P450 aromatase. The pattern of steroid receptor expression in the pTr2 cells, which is comparable to that shown for day 12F embryos, indicate that pTr2 cells maintain the same characteristics as the day 12F embryos collected in vivo.

To further characterize the function of ERβ in pTr2 cells, we determined the ability of ERβ to activate transcription of a reporter construct containing 4 copies of an ERE. ERβ in pTr2 cells activated transcription of the reporter gene in the presence of added E, suggesting this protein ability to interact with ERE in an E-dependent manner. The effect of E on transcriptional activity is mediated

through ERβ, since the use of an estrogen receptor antagonist was able to block the activation of the reporter construct. It was also observed that in cells under serum conditions, the transcriptional activity of thymidine kinase (tk)-promoter was increased independent of E. This result suggests that growth factors present in the serum are able to interact with the tk-promoter and/or ERβ to stimulate transcription. It is known that ER contains several conserved serine residues within the transactivation domain AF-1 that are targets for phosphorylation upon activation of MAP kinase pathways in the absence of E (Bunone et al., 1996). Also ligand-independent phosphorylation of ERβ can stimulate a recruitment of coactivators proteins with the subsequent increase in transcriptional activity (Tremblay et al., 1999).

Since pTr2 cells are ERβ exclusive, we analyzed the interaction between endogenous ERβ and exogenous human ERα in a series of co-transfection experiments. In cells under serum free conditions, the interactions of the two receptors increase the basal transcription activity of the reporter construct independent of E. However, when fold inductions were analyzed, this suggested a partial antagonistic effect of hERα, since there was a decrease in transcriptional activity from 4-fold in the experiment with ERβ alone to 2-fold in the experiment where hERα expression construct was cotransfected into these cells. When the two receptors were together in the presence of serum factors, there was a reduction with the basal transcription activity of the reporter construct. This reduction was independent of the presence of E, but was dependent on serum factors and hERα. Comparison of the fold induction in cells treated with E alone, and in those cells treated with E and cotransfected with the hERα expression construct, showed no change in fold induction with E. In every experiment where the cells received the hERα expression construct

under serum conditions, there was a decrease in transcriptional activity of the reporter construct. We know that ERα and ERβ can form heterodimers and these complexes can bind coactivators to stimulate transcription (Cowley et al., 1997; Pettersson et al., 1997). Also the transcriptional activity of the heterodimers lie between ERα and ERβ homodimer activity levels (Cowley et al., 1997; Ogawa et al., 1998). The explanation for the responses observed in our studies could be that ERα is antagonizing the action of ERβ to activate transcription and the presence of serum factors are potentiating that antagonistic effect. This could be the result of a phosphorylation of the receptors that can produce changes in the structure of the dimers which are not favorable to the formation of a stable pre-initiation complex, therefore the transcription capability is reduced.

Our studies also indicate that ERβ-mediated DNA synthesis in the pTr2 cell line which exhibited decreased DNA synthesis when treated with E under serum-free conditions. Similarly a day 14 trophectoderm cell line Jag-1 had decreased DNA synthesis with added E. However, in pTr2 cells under serum conditions, the reduction in DNA synthesis produced by E was no longer observed. This suggests that growth factors present in the serum can act on the cells and override the negative effects of E on cell growth. It is well documented that periimplantation porcine embryos express growth factor receptors (Kennedy et al., 1994; Green et al., 1995), and recently pTr2 cells were shown to be responsive to the action of keratinocyte growth factor (KGF), which stimulates the proliferation of these cells (Ka et al., 2001). Even though the uterine luminal fluids contain growth factors that can override the effect of E in decreasing cell growth, E is not the only steroid in the luminal fluids, and the concentration and composition of serum factors are different than in those of luminal fluids.

Therefore, the observation of decrease DNA synthesis by E in these embryonic cells is important for the understanding E-ERβ function in the process of embryo development.

The action of ERβ in decreasing cell growth has been previously observed. During vascular injury, when cells are proliferating rapidly, ERβ acts to decrease cell growth (Lindner et al., 1998). ERβ also has been associated with slowly proliferating breast cancer (Jarvinen et al., 2000), and interacting with proteins involved in the progression of mitosis such as MAD2, which function as a mitotic spindle assembly check point (Poelzl et al., 2000). In the previous chapter we described how ERβ expressed by embryos upon binding of E in culture was able to decrease the expression of PCNA, a gene correlated with growth.

In conclusion, pTr2 cells exhibit similar characteristics to day 12F embryos with respect to the steroid receptor gene expression. Moreover, the physiological functions observed in pTr2 cells corroborate the data obtained from Chapter 3 where E targets embryonic tissues mainly through ERβ. One potential functional consequence of E binding to ERβ in embryonic tissues is the control of embryonic cell growth. This action of ERβ-mediated E-signaling may underlie the high rate of embryo mortality in pigs, specifically in European breeds whose embryos secrete higher levels of estrogens than those of the Chinese breed (Ford, 1997).

CHAPTER 5
MODULATORY ROLE OF ANDROGEN RECEPTOR IN THE PORCINE ENDOMETRIUM DURING PERI-IMPLANTATION

Introduction

The pig uterine endometrium undergoes morphological changes during early pregnancy under the influence of steroid hormones (Knight et al., 1974). Receptors for progesterone (P) and estrogen (E) are known to be present in the endometrium during peri-implantation (Geisert et al., 1993), and upon stimulation by P and E, the uterus produces and secretes histotroph required for embryo development and success of implantation (Roberts and Bazer, 1988; Roberts et al., 1993; Simmen et al., 1995). Much less is known about the role of androgens or the androgen receptor (AR) in the porcine endometrium, or about any of its potential roles in controlling uterine receptivity. However, androgens are known to be present in uterine tissues and in circulation of mammalian females (Bonney et al., 1984; Stone and Seamark, 1985). Androgen binding sites have also been detected in mammalian uterine tissues (Heyns et al., 1976). The receptor for these androgens is a member of the superfamily of nuclear receptors. The human AR gene is localized to the X chromosome and encodes a 110 kDa protein composed of 919 amino acids (Chang et al., 1988).

Although AR knockout mice have not yet been generated, androgens in the female ERα knockout mice have been shown to increase uterine wet weight (Couse and Korach, 1999). AR is present in the nucleus of both stromal and epithelial cells in human endometrial tissue of the cycle (Horie et al., 1992). In human endometrial cells, androgens mediated by AR have been shown to stimulate prolactin secretion (Narukawa et al., 1994). Estrogen and androgens also stimulated IGF-I mRNA expression in uterus from ovariectomized rats. However, the effects of estrogen and androgens are quite different (Sahlin et al., 1994). Estrogen rapidly increased IGF-I expression for a short period of time whereas androgens maintained the levels of IGF-I mRNA at high levels for a longer time period (Sahlin et al., 1994). Levels of AR mRNA are increased by a combination of estradiol and testosterone in the primate uterus (Adesanya-Famuyima et al., 1999). In the endometrial carcinoma cell line Ishikawa, treatment with estradiol increased AR mRNA expression, while treatment with progesterone had the opposite effect. However, treatment with DHT and testosterone had a modest effect in increasing AR mRNA levels (Lovely et al., 2000). Another important observation is that testosterone and DHT displayed an antiestrogen effect in the endometrium by decreasing the expression of estrogen-regulated genes (Lovely et al., 2000), and high levels of androgens and AR have been associated with poor reproductive performance in women (Okon et al., 1998).

Androgens are present in the uterine fluids of pregnant pigs during the time of peri-implantation (Stone and Seamark, 1985). These androgens are

believed to serve as P450 aromatase substrates for estrogen production. Also, peri-implantation embryos, through the action of P450 type III aromatase, are likely to produce 19-norandrogens (Kao et al., 2000). However, there are currently no reports to demonstrate the presence of AR in the porcine uterus and to document their possible functions in uterine gene regulation. In this study, we determined the presence of the AR mRNA in uterine endometrial cells and the pattern of its expression throughout pregnancy in the pig. We also demonstrated the functionality of this receptor and the importance of the interactions between androgen and estrogen in regulating endometrial gene expression and cell growth.

Materials and Methods

Materials

TRIzol, Minimal Essential Medium (MEM), and MEM vitamin solution were purchased from Gibco BRL. Antibiotic-antimycotic (ABAM), Fetal Bovine Serum (FBS), RPMI 1640, estradiol 17β, testosterone and 19-nortestosterone were purchased from Sigma Chemical Co. [α-^{32}P] Deoxycytidine triphosphate (specific activity 3000 Ci/mmol), [^{3}H] Thymidine (specific activity 6.7 Ci/mmol) and BioTransnylon membranes were purchased from ICN Biotech. All molecular biology-grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Animals

All animal-use procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Prepubertal gilts were monitored daily for estrus detection. At the onset of estrus, gilts were mated with mature boars. The day of estrus was considered as day 0 of the cycle. Gilts were slaughtered at days 10 and 12 of pregnancy at the University of Florida Meats Processing Facilities. Reproductive tracts were collected and immersed in ice. Uterine horns were taken to a laminar flow hood, where they were flushed with PBS-2% ABAM to remove conceptuses, and endometrium was trimmed out and used for explant and primary cell cultures.

Primary Cell Culture

Glandular epithelial (GE) and stromal (ST) cells were isolated from days 10 and 12 pregnant uterine endometrium and cultured as previously described (Reed et al., 1996). Primary cells were grown in RPMI 1640 supplemented with 10% FBS, 0.25 U/ml insulin, and 1% ABAM. Medium was changed every other day. At 90-95% confluence, cells were washed with HBSS and cultured in serum-free, insulin-free RPMI 1640. After 24 h of incubation, fresh medium supplemented with the corresponding treatments were added to the cells. Incubation was then carried out for another 24 h at 37 ^{o}C under an atmosphere of 95% air, 5% CO_2.

Uterine Explant Cultures

Endometrial explants were isolated and cultured as previously described by Green et al. (1998). Uterine horns from days 10 and 12 pregnant pigs were opened, endometrial tissues were collected and washed in PBS-2% ABAM, and then minced into 1-3 mm^3 sections. Tissues (250 to 300 mg) were cultured in 3 ml of MEM supplemented with nonessential amino acids, glutamine (292 mg/L), MEM vitamin solution (10 ml/L), glucose (3.0 g/L) and 1% ABAM in 6-well tissue culture plates on a rocking platform at 37°C under an atmosphere of 50% N_2-47.5% O_2:2.5% CO_2. After 2 h pre-incubation, medium was changed to fresh medium supplemented with the following treatments: 17β estradiol (E) (50 nM); testosterone (T) (50 nM); 19-nortestosterone (N) (50 nM); E+T (50 nM each); E+N (50 nM each). The explants were further cultured for 24 h, collected and stored at -80 °C until used for Northern Blot analysis.

RNA Isolation and Analysis

Total cellular RNA was isolated from uterine endometrium on days 10, 12, 14, 18, 30, 60, and 90 of pregnancy, and from uterine endometrial explants using TRIzol reagent according to the manufacturer's specifications. Also, total RNA was obtained from uterine explants treated with E, T, N, E+T and E+N at a concentration of 50 nM each for 24 h. Northern blot analysis was performed as previously described in Chapter 3. All membranes were hybridized to nick translated cDNA fragments corresponding to porcine androgen receptor (AR) (283 bp), porcine GAPDH (593 bp), porcine PCNA (520 bp), human IGF-I (252

bp), and porcine ornithine decarboxylase (ODC)(263 bp). Hybridization signals were detected by autoradiography and quantified by densitometric analysis using an Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Co. San Leandro, CA). Membranes were stripped in between subsequent hybridization with one of the probes indicated above. RT-PCR was performed using cDNAs prepared from RNA collected from day 12 glandular epithelial (GE), luminal epithelial (LE) and stromal (ST) cells. PCR was performed as previously described in chapter 3. The total reaction for each PCR contained 50 pmol of AR forward and reverse primers (5' TCTTTGATGAACTTCGAATG 3'; 5' AATAGATGGGCTTGACTTTC 3'), 10 nM of each of the dNTPs, 1X PCR buffer (10 mM $MgCl_2$, pH 9.0) and 1 U of Taq polymerase in a total volume of 50 µl. PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide, and were visualized under UV light.

Thymidine Incorporation Assays

Cells were grown to confluence in 6-well culture dishes and then incubated in the appropriate serum-free medium for 24 h as previously described in Chapter 4. Monolayers were washed with HBSS, and medium containing the indicated treatments (ratios of 17β estradiol to testosterone; 0:0, 0:50; 0:500, 50:0, 50:50, 50:500, 500:0, 500:50, and 500:500 nM in serum-free media) were added to each well. After 20 h, 1 µCi of [^{3}H]thymidine was then added per well. After labeling for 4 h, cells were washed with PBS, fixed with methanol and DNA was precipitated by two incubations with trichloroacetic acid (5% TCA) for 10

min. The precipitate was recovered using 300 µl of NaOH (0.3 M). Radioactivity incorporated into the TCA-precipitable material was determined by liquid scintillation counting.

Statistical Analysis

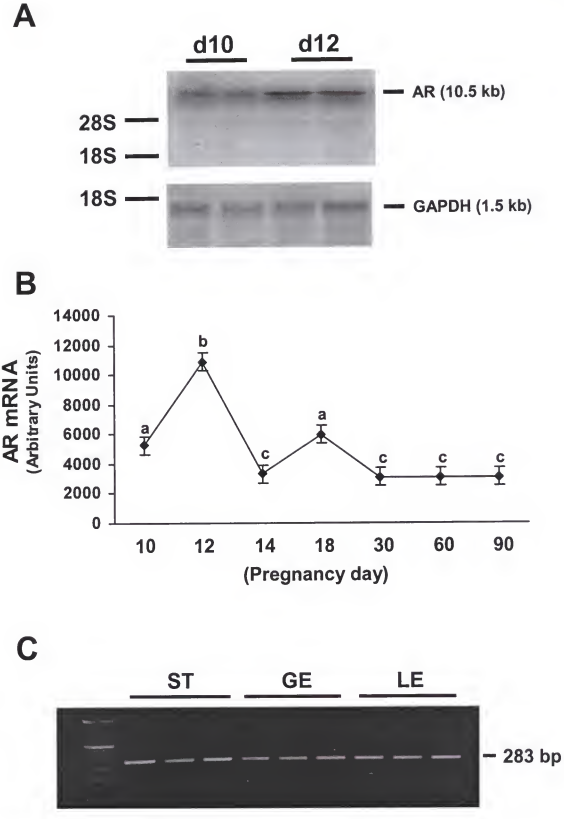
Data from the uterine explant experiments were subjected to least squares ANOVA using the General Linear Models procedure of SAS (Barr et al., 1979). The model tested was composed of two factors: treatment and experiment plus the interaction of treatment by experiment. Treatment was considered a fixed factor where experiment was considered random. For the thymidine incorporation assay the model was composed of three factors: 17β estradiol concentration, testosterone concentration, number of experiments, and the double and triple interactions between the three factors. These were also considered fixed except the experiment which was considered random factor. Values were considered significant at $p<0.05$ and are presented as least squares means (LSM)$\pm$ SEM.

Results

Characterization of AR Gene Expression in the Pregnant Pig Uterus

Total RNA prepared from endometrial tissues collected on days 10,12, 14, 18, 30, 60 and 90 of pregnancy from three individual pigs for each stage were used in Northern Blot analyses, using a radiolabeled porcine AR probe (283 bp).

Fig. 5-1. Levels of AR transcripts in endometrium during pregnancy and in primary cell cultures of GE and ST cells of a day 12 pregnant pig endometrium. A, Total cellular RNA obtained from endometrial tissues isolated from days 10 and 12 of pregnancy were analyzed by Northern Blot hybridization using an AR probe as described in Materials and Methods B, Total cellular RNA obtained from endometrial tissues isolated from days 10, 12, 14, 18, 30, 60 and 90 of pregnancy were analyzed by Northern Blot hybridization using an AR probe (30 µg of total RNA per sample, and 3 pigs per pregnancy stage). Densitometric values for each band, normalized to that for corresponding GAPDH were used for statistical analysis. Values with different superscripts are significantly different ($P<0.05$). C, Primary GE, ST and LE cells were analyzed for expression of AR mRNA by RT-PCR. A photograph of ethidium bromide-stained gels, where each lane was loaded with 20 µl of the resultant PCR reaction is shown. Each three lanes represent cDNA samples from GE, ST and LE cells respectively, isolated from day 12 pregnant porcine endometrium. The size of the PCR fragment for AR is 283 bp.


A
d10
d12
AR (10.5 kb)
28S
18S
18S
GAPDH (1.5 kb)
B
AR mRNA
(Arbitrary Units)
14000
12000
10000
8000
6000
4000
2000
0
a
b
c
a
c
c
c
10
12
14
18
30
60
90
(Pregnancy day)
C
ST
GE
LE
283 bp

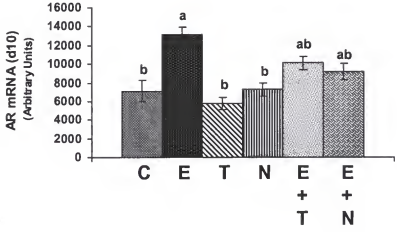
As shown in Figure 5-1A, a single transcript of approximately 10.5 kb in size corresponding to AR transcript previously reported (Brown et al., 1989) was detected in endometrial samples. The expression of AR during pregnancy was detected on day 10, increased by day 12, and decreased again by day 14. A significant increase from day 14 levels was observed to day 18, but this expression decreased to almost undetectable levels from day 30 until late pregnancy (Fig 5-1B). The cell distribution of AR mRNA expression was determined by RT-PCR using mRNA from cultured GE, ST and LE cells. The primers used in this analysis amplified a region of 283 bp within the AR cDNA, and were located in different exons to ensure the absence of false results arising from genomic DNA contamination. The presence of AR mRNA was detected in all three cell types (Fig. 5-1C).

Effect of Estrogen and Androgens on Uterine Gene Expression

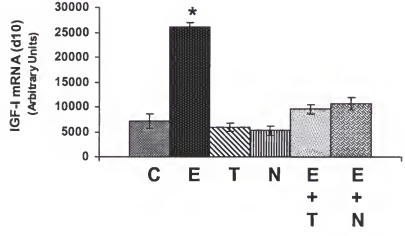
Since uterine luminal fluids contain estrogen and androgens during the time of implantation (Stone and Seamark, 1985), we examined the effects of estrogen and androgens alone and in combination, on uterine gene expression. Days 10 and 12 pregnant endometrial explant were incubated for 24 h with E, T, N and the combinations of E+T and E+N. On day 10 of pregnancy, AR mRNA was significantly stimulated by E treatment; however, when androgens were present in the media in combination with E, AR levels decreased relative to those obtained with E-treatment alone but were above those obtained with either androgen alone. Androgens (T or N) did not have any effect on AR gene

Fig. 5-2. Effect of steroid hormones on endometrial gene expression. Uterine endometrial explants from day 10 pregnant pigs were cultured in the presence or absence of E, T, N and the combinations of E+T and E+N. A, AR mRNA levels for each treatment. B, IGF-I mRNA levels for each treatment. Results represent least-squares means ± SEM of 3 experiments, in which each experiment represents endometrial tissue collected from a different pig, and for each pig, 3 replicate treatments. Densitometric values for each band, normalized to that of corresponding GAPDH were used for statistical analysis. Columns with different superscripts and * are significantly different ($P<0.05$).

A


AR mRNA (d10)
(Arbitrary Units)
16000
14000
12000
10000
8000
6000
4000
2000
0
a
b
b
b
ab
ab
C
E
T
N
E + T
E + N

B


IGF-I mRNA (d10)
(Arbitrary Units)
30000
25000
20000
15000
10000
5000
0
*
C
E
T
N
E + T
E + N

expression (Fig. 5-2A). E significantly (>5 fold) stimulated the expression of IGF-I gene relative to untreated explants, but this effect of E was abolished by the co-addition of androgens (Fig 5-2B). The mRNA levels for PCNA, a gene correlated with growth were also significantly stimulated by E, and no induction above control levels were observed with androgens. The combination of E plus either androgen reduced the effect seen with E (Fig. 5-3A). The mRNA levels for ODC, a gene shown to be regulated by testosterone in other tissues (Cintron et al., 1996), were unaffected by E or androgens (Fig. 5-3B).

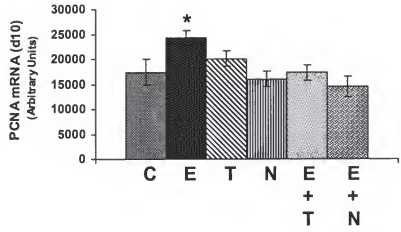
Neither estrogen nor androgens had any effect on the levels of AR mRNA in day 12 pregnant pig endometrium (Fig. 5-4A). The combination of E with either androgen showed a slight increase in the levels of AR mRNA, but the increase was not significant. When IGF-I mRNA levels were analyzed, it was observed that the combinations of E+T and E+N significantly increased the expression of the IGF-I gene over those of control levels (Fig. 5-4B). E alone had no effect. ODC mRNA levels were not changed either by E or T, but the combination of E+N had a small but significant stimulatory effect (Fig 5-5B). An induction of mRNA levels for PCNA was not detected in any of the steroid treatments, but instead a trend toward a reduction in expression under all treatments was observed (Fig 5-5A).

Effect of E and T on ^{3}H-thymidine Incorporation

To examine the effect of E or T on endometrial cell proliferation, GE and ST cells were isolated from days 10 and 12 pregnant pig endometrial tissues.

Fig. 5-3. Effect of steroid hormones on endometrial gene expression. Uterine endometrial explants from day 10 pregnant pigs were cultured in the presence or absence of E, T, N and the combinations of E+T and E+N. A, PCNA mRNA levels for each treatment. B, ODC mRNA levels for each treatment. Results represent least-squares means ± SEM of 3 experiments, in which each experiment represents endometrial tissue collected from a different pig, 3 replicate treatments. Densitometric values for each band, normalized to that of corresponding GAPDH were used for statistical analysis. Columns with * are significantly different ($P<0.05$).

A


PCNA mRNA (d10)
(Arbitrary Units)
30000
25000
20000
15000
10000
5000
0
*
C
E
T
N
E + T
E + N

B

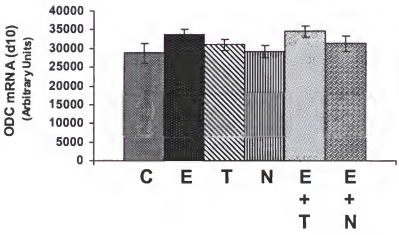
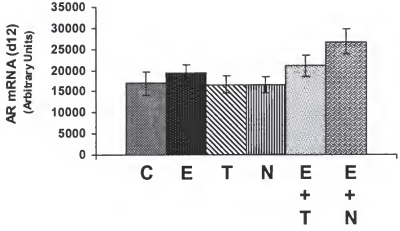

ODC mRNA (d10)
(Arbitrary Units)
40000
35000
30000
25000
20000
15000
10000
5000
0
C
E
T
N
E + T
E + N

Fig. 5-4. Effect of steroid hormones on endometrial gene expression. Uterine endometrial explants from day 12 pregnant pigs were cultured in the presence or absence of E, T, N and the combinations of E+T and E+N. A, AR mRNA levels for each treatment. B, IGF-I mRNA levels for each treatment. Results represent least-squares means ± SEM of 3 experiments, in which each experiment represents endometrial tissue collected from a different pig, and for each pig, 3 replicate treatments. Densitometric values for each band, normalized to that of corresponding GAPDH were used for statistical analysis. Columns with * are significantly different ($P<0.05$).

A


AR mRNA (d12)
(Arbitrary Units)
35000
30000
25000
20000
15000
10000
5000
0
C
E
T
N
E + T
E + N

B

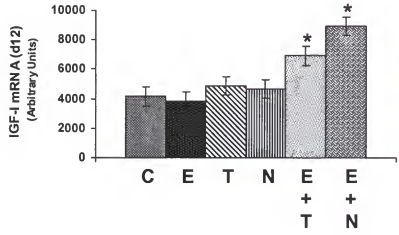
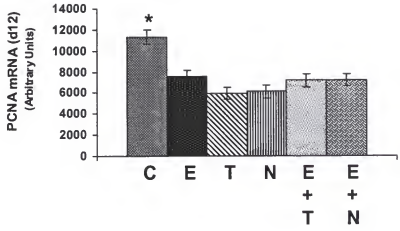
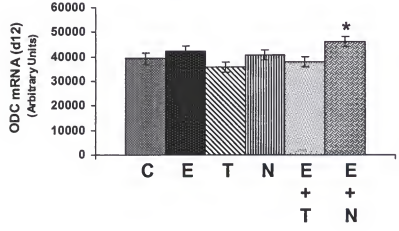

IGF-I mRNA (d12)
(Arbitrary Units)
10000
8000
6000
4000
2000
0
*
*
C
E
T
N
E + T
E + N

Fig. 5-5. Effect of steroid hormones on endometrial gene expression. Uterine endometrial explants from day 12 pregnant pigs were cultured in the presence or absence of E, T, N and the combinations of E+T and E+N. A, PCNA mRNA levels for each treatment. B, ODC mRNA levels for each treatment. Results represent least-squares means ± SEM of 3 experiments, in which each experiment represents endometrial tissue collected from a different pig, 3 replicate treatments. Densitometric values for each band, normalized to that of corresponding GAPDH were used for statistical analysis. Columns with * are significantly different ($P<0.05$).

A


PCNA mRNA (d12)
(Arbitrary Units)
14000
12000
10000
8000
6000
4000
2000
0
*
C
E
T
N
E + T
E + N

B


ODC mRNA (d12)
(Arbitrary Units)
60000
50000
40000
30000
20000
10000
0
*
C
E
T
N
E + T
E + N

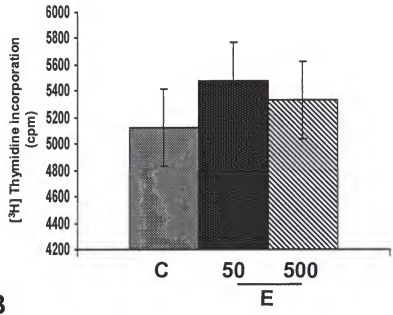
These cells were cultured to confluence and treated with different concentrations of E, T and the combination of E+T. For day 10 GE cells, a significant interaction between E and T was not observed; however, E alone at 50 nM numerically increased DNA synthesis compared to the controls (Fig. 5-6A). A numerically decrease on DNA synthesis by T at two concentration levels was also observed (Fig. 5-6B). When ST cells were analyzed, E and T treatments similarly did not show any significant interaction. Estrogen had no significant effect on ST DNA synthesis, however, there was a tendency to increase DNA synthesis with increasing E concentration (Fig. 5-7A). However, ST cells that received 50 nM of T had numerically decreased DNA syntheses compared to controls (Fig. 5-7B).

A significant interaction of E and T on DNA synthesis in day 12 GE cells was not observed. However, GE cells showed a numerically increase of DNA synthesis upon treatment with 500 nM of E (Fig 5-8A). An opposite response was observed when GE cells received 50 and 500 nM of T, respectively. In this case, cells had numerically decreased DNA synthesis compared to control cells (Fig 5-8B). With day 12 ST cells, a significant interaction between E and T on DNA synthesis was not observed. ST cells showed numerically decreased DNA synthesis when treated with 50 and 500 nM of T in the presence of high levels of E (500 nM)(Fig 5-9C). However at low levels of E (0 and 50 nM), T did not show any effect in decreasing DNA synthesis relative to control cells (Fig. 5-9A, B).

Fig. 5-6. Effect of 17β estradiol (E), and testosterone (T) on [^{3}H] thymidine incorporation. Primary GE cell cultures from a day 10 pregnant pig endometrium were treated with increasing concentrations of E (A) or T (B) as described in Materials and Methods. Results represent least-squares means ± SEM of 3 experiments, with each experiment representing endometrial GE cells collected from a different pig. For each pig, the number of replicate was 3 per treatment group.

GE (day 10)

A


[3H] Thymidine incorporation (cpm)
6000
5800
5600
5400
5200
5000
4800
4600
4400
4200
C
50
500
E

B

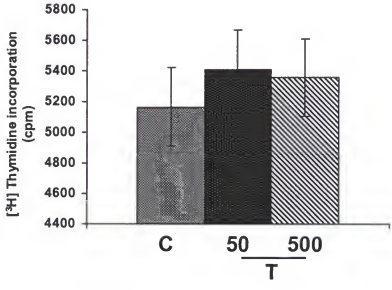
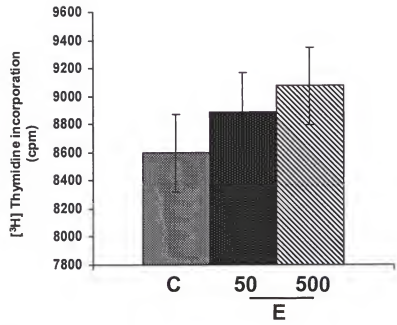

[3H] Thymidine incorporation (cpm)
5800
5600
5400
5200
5000
4800
4600
4400
C
50
500
T

Fig. 5-7. Effect of 17β estradiol (E), and testosterone (T) on [^{3}H] thymidine incorporation. Primary ST cell cultures from a day 10 pregnant pig endometrium were treated with increasing concentrations of E (A) or T (B) as described in Materials and Methods. Results represent least-squares means ± SEM of 3 experiments, with each experiment representing endometrial ST cells collected from a different pig. For each pig, the number of replicate was 3 per treatment group.

ST (day 10)

A


[3H] Thymidine incorporation (cpm)
9600
9400
9200
9000
8800
8600
8400
8200
8000
7800
C
50
500
E

B

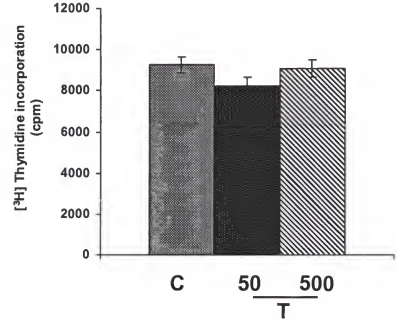
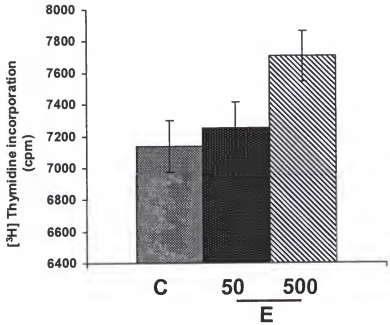

[3H] Thymidine incorporation (cpm)
12000
10000
8000
6000
4000
2000
0
C
50
500
T

Fig. 5-8. Effect of 17β estradiol (E), and testosterone (T) on [^{3}H] thymidine incorporation. Primary GE cell cultures from a day 12 pregnant pig endometrium were treated with increasing concentrations of E (A) or T (B) as described in Materials and Methods. Results represent least-squares means ± SEM of 3 experiments, with each experiment representing endometrial GE cells collected from a different pig. For each pig, the number of replicate was 3 per treatment group.

GE (day 12)

A


8000
7800
7600
7400
7200
7000
6800
6600
6400
[³H] Thymidine incorporation (cpm)
C
50
500
E

B

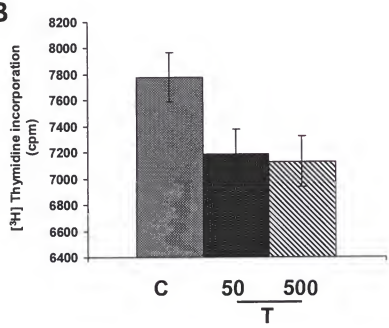
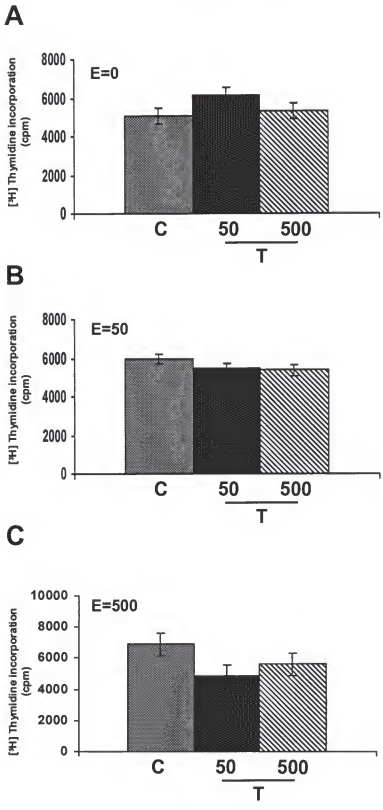

8200
8000
7800
7600
7400
7200
7000
6800
6600
6400
[³H] Thymidine incorporation (cpm)
C
50
500
T

Fig. 5-9. Effect of 17β estradiol (E), and testosterone (T) on [^{3}H] thymidine incorporation. Primary ST cell culture from a day 12 pregnant pig endometrium were treated with increasing concentration of T at 0 nM (A), 50 nM (B), and 500 nM (C) of E as described in Materials and Methods. Results represent least-squares means ± SEM of 3 experiments, with each experiment representing endometrial ST cells collected from a different pig. For each pig, the number of replicate was 3 per treatment group.

ST (day 12)


A
[³H] Thymidine incorporation (cpm)
8000
6000
4000
2000
0
E=0
C
50
500
T
B
[³H] Thymidine incorporation (cpm)
8000
6000
4000
2000
0
E=50
C
50
500
T
C
[³H] Thymidine incorporation (cpm)
10000
8000
6000
4000
2000
0
E=500
C
50
500
T

Discussion

Androgens are present in the uterine luminal fluids of several mammalian species (Bonney et al., 1984; Terada et al., 1993). In pigs, androgens are present in the uterine luminal fluids at peri-implantation (Stone and Seamark, 1985). A recent report has shown that P450 aromatase expressed by porcine trophoblastic cells is able to synthesize not only estrogen but also 19-nortestosterone (Kao et al., 2000). The relevance of these androgens in the porcine uterus is still unknown, however in other female mammals, AR has been localized to uterine cells (Muechler, 1987; Adesanya-Famugicia et al., 1999). Here, we report AR gene expression by the uterine endometrium during pregnancy. An AR transcript of approximately 10.5 kb consistent with previous reports for other species, was detected in uterine endometrial tissues (Brown et al., 1989). The highest expression appears to be on day 12 after fertilization, coincident with the peak levels of estrogen in the uterine lumen (Gadsby et al., 1980; Pusateri et al., 1990). By day 14, AR mRNA levels decreased, but on day 18 a small but significant increase in the levels of AR mRNA was observed. It is known that between days 15 to 18 there is an increase in estrogen content in uterine fluids, and this increase is required for pregnancy to be sustained until term (Geisert et al., 1990). It is possible that this second rise in estrogen levels in the uterine luminal fluids is responsible for the observed increase in AR mRNA levels, since it has been reported that human carcinoma endometrial cells had increased levels of AR mRNA when they are exposed to E (Lovely et al., 2000).

This is also in agreement with our results from the explant culture experiments. After day 30 of pregnancy the levels of AR mRNA were almost undetectable. Using RT-PCR analysis, we showed that AR transcripts were present in the three endometrial cell types GE, ST and LE. This analysis however, was not quantitative, hence we can not distinguish which cell type expresses higher levels of AR mRNA. However, a previous study from our laboratory (Vale-Cruz et al., 2001) showed the immunolocalization of AR protein predominantly to uterine endometrial GE and ST cells of day 12 pregnancy, with undetectable levels in LE cells.

In order to determine if endogenously expressing AR present in those cells is functional, we performed a series of experiments using endometrial tissues of days 10 and 12 pregnant pigs. One important difference between these two stages is that day 10 endometrial tissues have not been exposed to the high levels of estrogens secreted by the embryo beginning at day 11 of pregnancy. When we analyzed if AR mRNA levels were affected by exposure to E, T, N and the combination of E with N or T, we detected an up-regulation of AR mRNA levels by E. This increase by E has also been documented in the Ishikawa human endometrial carcinoma cell line (Fujimoto et al., 1994; Lovely et al., 2000). Our results did not show any effect of T or N on AR mRNA levels. It has also been reported in the Ishikawa cell line, that treatment with Dihydrotestosterone (DHT) or hydroxyflutamide, an inhibitor of AR action, had no effect on AR mRNA levels (Lovely et al., 2000). Interestingly, the treatment combination of E + N decreased AR mRNA levels from that of E-treated

explants. The lack of effect of T on E-induction of AR mRNA levels has also been previously reported (Lovely et al., 2000). The influence of N on E could be due to the utilization by N of a different receptor from T, since N has been shown to bind ERβ for biological action (Kuiper et al., 1997). This ER isoform is expressed in the uterus of pregnant female mice and can modulate the effect of E acting through ERα to decrease cell proliferation (Weihua et al., 2000). In this regard, preliminary studies in our laboratory using RT-PCR demonstrated presence of ERβ transcript in early pregnancy pig endometrium (Simmen RCM, unpublished data).

In the same set of experiments, we observed an up-regulation of IGF-I mRNA levels by E. In pigs, an increase in E content in uterine luminal fluids has been correlated with increased IGF-I levels (Simmen et al., 1995). These data, taken together suggest a direct effect of E on uterine IGF-I production, although there is a recent report that E can stimulate IGF-I activity as well (Kahlert et al., 2000).

We did not observe any effect of androgens on IGF-I gene expression. However, the combination of E with either androgen (T or N) blocked the effect of E alone. This anti-estrogenic effect of androgens has also been reported in the Ishikawa human endometrial carcinoma cell line. Lovely et al. (2000) using alkaline phosphatase, a gene regulated by E, showed that induction of this gene by E was blocked by addition of DHT to the media. Another example of this inhibitory effect was demonstrated by Panet-Raymond et al. (2000) where the transactivational activity of ERα on a reporter gene was decreased by 60% by

AR.

Porcine uterine endometrium at early pregnancy and corresponding peri-implantation embryos express IGF-I and IGF-I receptor (Corps et al., 1990; Hofig et al., 1991), and the action of IGF-I in the uterine lumen is presumably to stimulate uterine growth (Simmen et al., 1995). A direct action of IGF-I on porcine embryo has not been clearly established to date, however, it has been demonstrated in vitro that IGF-I can stimulate expression of porcine embryo P450 aromatase gene (Green et al., 1995; Simmen et al., 1995). When we analyzed the effect of E on the expression of PCNA, a gene correlated with growth in the endometrium, and in other tissues and cell types we noted that PCNA mRNA levels were increased by E. However, when androgens (T or N) were added together with E, the effect of E on the expression of PCNA gene was blocked. This response is similar to that seen for IGF-I gene expression. Taken together these data suggest that the inhibitory effect of androgens on growth may be mediated through inhibition of E-induced IGF-I gene expression, and that inhibition of E-induced gene expression by AR-T complex has been shown to occur in vitro (Panet-Raymond et al., 2000).

We also analyzed the effect of androgens (T or N) on endometrial ornithine decarboxylase (ODC) gene expression which was previously shown to be increased by androgens in other cell and tissue systems (Cintron et al., 1996). None of the treatments used alone or in combinations had any effect on endometrial ODC gene expression. These results suggest that ODC regulation by androgens may be a tissue-specific phenomenon.

We repeated the experiments using day 12 pregnant pig endometrial tissues, and observed different responses from those seen with endometrial tissues of day 10 pregnant pigs. There was no effect of E, N, T and their combinations on AR mRNA levels in endometrial explants from day 12 pregnant pigs. When we analyzed for IGF-I gene expression, only the combination of E+T and E+N showed a significant effect. PCNA, in contrast, was significantly reduced by all treatments. When we analyzed the effects of the treatments on ODC mRNA levels, we detected a significant increase in expression by the combination of E+N. These responses were different from those obtained using day 10 pregnant pig endometrial tissues. These differences are likely due to the fact that day 12 endometrial tissues had already been exposed to E and possibly N, both of which are secreted by developing embryos (Pusateri et al., 1990; Kao et al., 2000). Also, many other components in the uterine luminal fluids reach maximal levels at day 12 of pregnancy; these include retinol and growth factors such as IGF-I (Simmen et al., 1992; Roberts et al., 1993). These compounds are present at much higher concentrations in the uterine environment at day 12 of pregnancy and likely modify the responsiveness of endometrial tissues to steroid hormones.

To further characterize the biological effect of E and/or androgens on endometrial tissues, we used primary cell cultures. Highly purified populations of GE and ST cells from day 10 and day 12 pregnant pig endometrial tissues were obtained following previously published procedures (Reed et al., 1996). A factorial experimental design was used to analyze the effects of E and T and

their combinations. In cells (GE, ST) collected from day 10 pregnant pig endometrium, a significant interaction effect between E+T treatments was not observed. However, these cells showed a positive response to added E at 50 nM concentration but was not statistically significant. GE cells treated with 500 nM of E, however did not show a significant increase in DNA synthesis, although a tendency towards an increase compared to the control cells was observed. When GE cells received T, no changes in DNA synthesis was observed. Interestingly ST cells in the presence of T showed a decrease in DNA synthesis. All these changes were not statistically significant. However, ST cells receiving E had no observable changes in levels of DNA synthesis. Thus, different cell types have distinct responses to steroid hormones. Consistent with this, the production of lactoferrin in mouse uterus was shown to be directly mediated in epithelial cells but not stromal cells by E (Tibbetts et al., 1998).

GE cells isolated from day 12 pregnant pig endometrium showed an increase in DNA synthesis with E (500 nM). However, these cells had decreased DNA synthesis with T. Interestingly in Day 12 ST cells, there was a negative effect of T on DNA synthesis observed at maximum E concentration but was not statistically significant. This response is likely physiologically relevant during the time of maternal recognition of pregnancy since decrease in uterine growth could conceivably allow the initiation of implantation. The lack of responses of the primary cell cultures compared to the significant responses observed in the explant culture suggested the important of cell-cell interaction in gene regulation by steroids

Results of the primary cell culture studies indicate that the overall effect of E could be antagonistic to that of T. Comparison of these results with those obtained from uterine explant experiments indicate very similar effects of E and T, since E increased IGF-I and PCNA gene expression while T decreased the levels of these mRNAs. The growth stimulatory effect of E can be mediated by IGF-I, since previous studies reported that ERα, the predominant ER isoform in the uterus, can activate the IGF-I signaling pathway (Kahlert et al., 2000). Also it has been reported that the stimulatory effect of E on gene expression can be blocked or modulated by the androgen-AR complex (Lovely et al., 2000; Panet-Raymond et al., 2000). Even more interesting is the possibility that 19-nortestosterone, produced by peri-implantation porcine embryos acts through ERβ to modulate the function of ERα in the uterus; the latter has been previously reported to occur in the mouse uterus, where ERβ modulates the effect of ERα by decreasing cell proliferation induced by E (Weihua et al., 2000). However, there are slight differences with some of the DNA synthesis responses in the primary cell experiments. The DNA synthesis in day 10 ST cells and corresponding GE cells treated with E and T respectively were not affected relative to those of control cells. Such distinct responses point out to the importance of the interaction between stromal and epithelial cells. This has been demonstrated by Buchanan et al. (1999), where both stromal and epithelial cells are required for estrogen induction of the proliferative response of uterine epithelium in mice.

In conclusion, the androgens present in porcine uterine luminal fluids at

early pregnancy do not solely serve as substrates for the production of E. They appear to have an important role in modulating the biological effects of high levels of E present in the uterine lumen at the time of peri-implantation. The ligand bound-AR and the E derived from embryonic source acting through its own receptors in the endometrium modulate endometrial gene expression and uterine growth which could be necessary for the initiation of the implantation process.

CHAPTER 6
SUMMARY AND CONCLUSION

Summary

The main objective of this dissertation is to understand the functional significance of estrogens produced by porcine embryos and androgens present in the uterine luminal fluids during the time of peri-implantation on embryonic development and maternal uterine gene expression. An understanding of the roles of these steroids may help explain the high rate of embryo mortality seen in pigs and provide insight for development of strategies to increase reproductive efficiency for improving pig production. Since estrogens produced by developing embryos act as maternal recognition of pregnancy signals and their concentrations in uterine luminal fluids are very high at peri-implantation, the first logical step was to determine if porcine embryos are targets of these estrogens and therefore, express functional estrogen receptors. Using multiple alignments of several ERβ sequences from different mammalian species, several primer sets were designed based on the conserved regions among those sequences. These primer sets were used in RT-PCR reactions using day 12F embryo mRNA as template. The results of these reactions were four overlapping cDNA fragments, which together comprise the full coding sequence of the porcine

embryo ERβ mRNA.

Using a fragment corresponding to the 5' region of the porcine embryo ERβ cDNA as a probe in Northern blots, we detected several mRNA transcripts using total RNA from day 12F embryos and poly A+ mRNA from day 12 pregnant pig endometrium. Four mRNA transcripts of 9.5 kb, 7.4 kb, 4.9 kb and 3.5 kb were identified. Size of these transcripts are in agreement with those previously described for mouse ERβ mRNAs (Tremblay et al., 1997).

Corresponding ERβ protein and its specific location in day 12F porcine embryo were demonstrated in the nucleus of trophectoderm cells using a rabbit antibody to rat ERβ. Gene expression analysis for ERα, ERβ, PR, cyclin D1, P450 aromatase Type III and GAPDH were carried out by RT-PCR analysis of embryos ranging in morphology from small spherical (5-6mm), to tubular (15 mm) to large filamentous (>100 mm) at distinct pregnancy days. ER-β gene expression was low in small spherical embryos (5-9 mm), increased progressively in larger embryos, and reached maximal levels at day 12F embryos, with a decline observed in day 14F embryos. In contrast, ERα gene expression was almost undetectable and remained so for embryos of all morphologies tested. PR mRNA expression was low in small spherical embryos, increased in tubular embryos, and was significantly diminished in F embryos. The mRNA levels for cyclin D1, a gene positively correlated with growth, were low in small spherical embryos, increased significantly ($P<0.05$) by the tubular stage, and reached maximal levels in d12F embryos, with a significant ($P<0.05$) decline observed by d14F. It is important to mention that highest ERβ and cyclin

D1 gene expression was coincident with the peak levels of estrogen in uterine luminal fluids. P450 aromatase Type III gene expression gradually increased from small to large spherical embryos, reached peak levels for tubular embryos, and decreased in d12F embryos to nearly undetectable levels by d14F. GAPDH transcript levels were relatively constant in all embryo morphologies and were thus, used in densitometric analysis to normalize the expression of other embryonic genes.

The functionality of embryonic ERβ was determined by in vitro culture experiments, using day 12F embryos treated with 17β estradiol (E) for 24 hours. ERβ and P450 aromatase type III gene expression were significantly ($P<0.05$) increased by treatment with E, however, the expression of the PCNA gene, whose corresponding gene product is correlated with growth, decreased significantly ($P<0.05$) after exposure to E. Interestingly, cyclin D1 gene expression was not affected by similar E treatment. These results demonstrate that the major estrogen receptor expressed by the embryos, namely ERβ, mediates E-effects on this tissue's gene expression. These data represent the first report of autocrine effects of embryonic-derived E mediated by ERβ for any mammalian species and raise the interesting possibility that embryonic growth is regulated in part, by the activation of the E-ERβ complex, since this complex down-regulated the expression of PCNA, a protein essential for DNA replication.

To further demonstrate a role for E in embryo function, pTr2 cells, a newly developed cell line from day 12F trophectoderm cells, was used. The expression of several steroid receptors and other genes by day 12F embryos (Chapter 3)

were evaluated in pTr2 cells using RT-PCR. The pattern of expression of ERα, ERβ, and PR genes were comparable between pTr2 cells and day 12F embryos. However, the P450 aromatase gene was expressed by day 12F embryos but not by pTr2 cells; this was not surprising since day 12F embryos when cultured for 24 h, lacked P450 aromatase gene expression.

To further characterize pTr2 cell ERβ expression, the presence of the ERβ protein was examined in these cells by using immunofluorescence. A strong immunofluorescence signal for ERβ was detected in the nuclei of pTr2 cells. By contrast, ERα protein in these cells was not detected following the same immunodectection protocol using a rat anti-human antibody . To demonstrate the specificity of the rabbit anti-rat ERβ antibody used in immunofluorescence studies, cells were incubated with the antibody that was pre-incubated with an ERβ immunizing peptide. The nuclear signal obtained with the anti-ERβ antibody was diminished by the presence of the immunizing peptide, indicating that the nuclear signal generated by anti-ERβ antibody is specific to porcine ERβ protein.

To determine the functionality of ERβ in pTr2 cells, a series of transfection experiments were performed using an E-responsive Luc reporter gene construct containing 4 copies of the ERE linked to the thymidine kinase promoter. Transiently transfected cells treated with E showed an increased transcriptional activity of the reporter gene construct relative to control (untreated) cells. The effect of E on reporter gene activity was inhibited by using a known ER antagonist (ICI 182,780). The transcriptional activity of the E-responsive reporter

gene construct was compared in the presence or absence of serum in culture medium. The added serum increased transcriptional activity of the reporter gene, independent of the presence of E. This result suggested two possibilities: (1) that transcriptional activity of the thymidine kinase promoter was increased by serum factors acting through specific serum responsive elements in the promoter constructs, or (2) that some serum factors are activating ERβ independent of the ligand. Additional experiments will be needed to clarify between those possibilities.

We also examined through cotransfection experiments the possibility of a functional interaction between endogenous ERβ and transfected human ERα in pTr2 cells. Exogenous hERα decreased the induction by E of the transcriptional activity of the reporter gene construct under serum-free condition. Similar experiments carried out under charcoal-stripped serum showed a reduction in the basal transcriptional activity of the reporter construct. In all experiments where hERα and serum factors were present, transcription from the reporter construct was decreased. These results could provide a physiological relevance for undetectable levels of ERα in the porcine embryos, since presence of ERα could result in an inhibition of E-mediated gene transcription.

pTr2 cells were used in mitogen assays to further examine the function of E on cell growth. These cells were treated with different concentrations of E (10, 50 and 500 nM) under serum-free conditions. DNA synthesis was decreased by the action of E at the higher concentrations tested (50 and 500 nM). Interestingly, the reduction on DNA synthesis was not observed when the

treatments were done in the presence of charcoal-stripped serum. A day 14 porcine trophectoderm cell line Jag-1, which also expresses ERβ similar to that of Day 14F embryos, was also used in mitogen assays to confirm the results observed with pTr2 cells. Treatment of Jag-1 cells with E decreased DNA synthesis. These results suggest that the reduction by E of PCNA gene expression previously observed in cultured d12F embryos is a consequence of E-inhibition of DNA synthesis in embryonic-derived cells that are mediated by ERβ.

Data in the literature reported the presence of androgens in uterine luminal fluids (Stone and Seamark, 1985), and that porcine P450 aromatase type III can catalyze the synthesis of 19-nortestosterone (N) (Kao et al., 2000). The presence of AR was determined in porcine uterine endometrium as a function of pregnancy (Chapter 5). Northern blot analysis of pregnancy endometrium using a porcine AR cDNA probe demonstrated that the levels of the 10.5 kb transcript corresponding to AR mRNA were higher at early than at mid- and late pregnancy stages. RT-PCR analysis using cDNA from the three cell types of early pregnancy uterine endometrium, namely GE, LE and ST showed the presence of AR transcripts. However, the RT-PCR methodology was unable to discriminate which cell type expressed highest levels of the AR gene.

To determine the functionality of the AR in the endometrium, endometrial explants from days 10 and 12 pregnant pigs were treated with E, testosterone (T), N, and the combinations of E+T and E+N, and the expression of several

genes were analyzed by Northern blot. Day 10 endometrial explants showed a significant ($P<0.001$) increase in AR gene expression with E, and with the combinations of E + T or N. IGF-I gene expression was also increased by E but E+T or E+N treatments suppressed the E effect. PCNA gene expression was increased by E, and the combination of E with T or N inhibited the individual stimulatory effect of E. Neither androgen treatments alone (T and N) had any effect on the expression of these genes. ODC, a gene regulated by T in prostate cells (Cintron et al., 1996) did not show any change in expression in day 10 endometrial explants after steroid treatments. The response of day 12 pregnancy endometrial explants to steroids was different from that of day 10 explant cultures. AR and ODC gene expression were not affected by any of the treatments. IGF-I gene expression was increased only by the combination of E with T or N, and PCNA gene expression was reduced by all treatments. These differences in responses between days 10 and 12 pregnancy endometrial explants may be due to the exposure in vivo of endometrial tissues to steroids secreted by the embryo after day 10 of pregnancy.

Primary GE and ST cells from days 10 and 12 pregnancy endometrium were used to characterize the effects of E and T alone and in combination, on DNA synthesis. E stimulated DNA synthesis in GE cells isolated from both days of early pregnancy. T had no effect on GE cells from day 10, but GE cells from day 12 had decreased DNA synthesis when treated with T. ST cells from day 10 showed a reduction in DNA synthesis when they were treated with T, but treatment with E had no effect on these cells. ST cells from day 12 showed an

interaction effect between E and T. In particular, treatment with T at the highest levels of E (500 nM) had a negative effect on DNA synthesis.

Conclusions

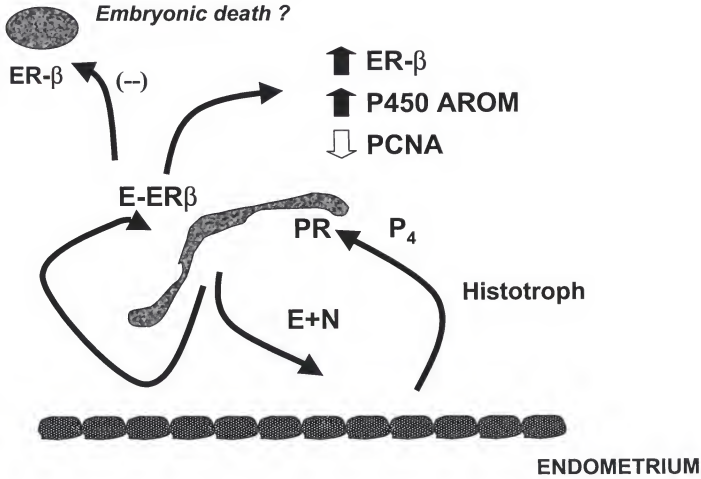
The peri-implantation period represents a very dynamic period for embryo development. It is characterized by dramatic changes in uterine gene expression and protein secretions (Roberts et al., 1993; Simmen et al., 1995). Porcine embryos synthesize and secrete estrogens, the maternal recognition signal for pregnancy, between days 11 and 12 (Gadsby et al., 1980; Pusateri et al., 1990). Also during this period, a high incidence of embryo mortality occurs (Pope, 1985). It has been postulated that estrogens secreted by the more advanced embryos target the uterus, causing significant changes in the uterine environment that are not optimal for less developed embryos (Morgan et al., 1987; Pope et al., 1990; Roberts et al., 1993). Reports showing a direct effect of E via ER-mediated action are limited in mammalian embryos and have been mostly demonstrated in mouse and rodents (Hou and Gorski, 1993; Hou et al., 1996; Ying et al., 2000); in pigs reports on the expression of ER by developing embryos are conflicting (Yelich et al., 1997; Dekaney et al., 1998).

The research presented in this dissertation showed for the first time that estrogens secreted by developing peri-implantation embryos can not only influence maternal uterine functions, but corresponding embryonic functions as well. The identification of ERβ mRNA and protein in the nucleus of

trophectoderm cells, and the pattern of ERβ gene expression, which is coincident with that of cyclin D1 suggest a role for ERβ in embryo development. However, since E did not increase expression of the cyclin D1 gene in vitro, the increase in cyclin D1 observed in vivo coincident with embryonic elongation is likely the result of increasing IGF-I concentrations in the uterine lumen (Ko et al., 1994). IGF-I has been shown to act on the porcine embryo through the IGF-I receptor, and this likely results in the stimulation of embryonic growth (Green et al., 1995; Simmen et al., 1995).

One of the actions of E on d12F embryos was to decrease PCNA gene expression. This together with the finding that E decreased DNA synthesis in porcine trophectoderm cell lines suggest a role for the E-ERβ complex in the regulation of embryonic growth (Fig 6-1). It has been reported that ERβ functions to slow growth in rapidly growing tissues (Iafrati et al., 1997; Lindner et al., 1998). Also, a recent report showed that ERβ physically interacts with proteins involved in the regulation of the cell cycle such as MAD2, a check-point molecule at mitosis (Poelzl et al., 2000). Embryonic-secreted E thus, appears to have a dual function: 1) it acts on the uterus to stimulate uterine protein synthesis and secretion (Roberts et al., 1993, Simmen et al ., 1995), including those of important growth-regulatory factors such as IGF-I (Chapter 5), and 2) it activates ERβ in the embryo to control the growth of this tissue. Additional actions of E on embryonic gene expression are the induction of ERβ and P450 aromatase type III gene expression, both of which are correlated with embryonic development. Our studies represent the first report of E regulation of P450

Fig. 6-1. Proposed model for autocrine and paracrine actions of embryonic estrogen. Arrows (⇑⇓) indicate increased or decreased levels of gene expression. Progesterone (P_4), estrogen (E), 19-nortestosterone (N), estrogen receptor β (ERβ), proliferative cellular nuclear antigen (PCNA), P450 aromatase (P450 AROM).

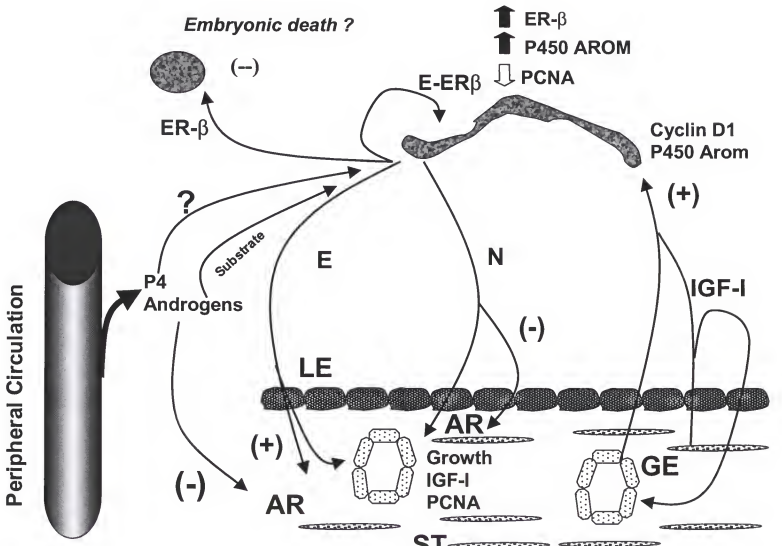

Embryonic death ?
ER-β
(--)
ER-β
P450 AROM
PCNA
E-ERβ
PR
P_4
Histotroph
E+N
ENDOMETRIUM

aromatase gene expression in porcine embryos.

Since developing embryos in vivo do not grow in synchrony, the data presented here might explain a natural selection process occurring in the uterus, whereby more developed embryos, through their production of estrogen, inhibit the growth of less developed embryos expressing ERβ (Fig. 6-1). Consistent with this, a breed of pigs known for its low embryonic mortality (Meishan) is characterized by lesser asynchrony in embryo development (Ford, 1997) and lower embryonic production of estrogens (Wilsom and Ford, 1997).

To add more complexity to the embryo-uterine interaction model (Fig. 6-2), the present studies demonstrated the expression of the AR gene in the porcine uterine endometrium and its possible role in regulating expression of E-stimulated genes. Previous work has shown AR gene expression in mammalian uterine cells (Muechler, 1987; Adesanya-Famugicia et al., 1999), and the inhibitory effects of androgens on E-regulated genes (Lovely et al., 2000). It is believed that uterine luminal androgens come from the circulation (Bonney et al., 1984; Stone and Seamark, 1985). However, a recent report indicated the ability of porcine embryonic P450 aromatase (type III) to produce not only estrogens but also N (Kao et al., 2000). Our results that androgens can modulate E-induced IGF-I and PCNA gene expression suggest the antiproliferative role of androgens in uterine growth (Fig. 6-2). This is in agreement with previous experiments carried out in non-human primates where androgen-AR mediated action was shown to be antiproliferative in endometrial growth (Slayden et al., 2001), and this effect could be linked to infertility problems that are related to

Fig. 6-2. Proposed model for the roles of steroids in embryo-uterine communication during peri-implantation. Arrows (⇑⇓) indicates increased or decreased levels of gene expression. Insulin like-growth factor-I (IGF-I), Progesterone (P_4), estrogen (E), 19-nortestosterone (N), androgen receptor (AR), estrogen receptor β (ERβ), proliferative cellular nuclear antigen (PCNA), P450 aromatase (P450 AROM), glandular epithelial (GE), luminal epithelial (LE), and stromal (ST) cells.


Embryonic death ?
(--)
ER-β
ER-β
P450 AROM
PCNA
E-ERβ
Cyclin D1
P450 Arom
(+)
?
Substrate
P4
Androgens
E
N
IGF-I
(-)
Peripheral Circulation
LE
AR
(+)
Growth
IGF-I
PCNA
GE
(-)
AR
ST

elevated androgen concentrations and androgen receptors within the human uterus (Okon et al., 1998).

Also, there is the possibility that N can bind to ERβ in the embryo as well as in the endometrium since this ligand has been shown to exhibit low, but specific affinity for ERβ (Kuiper et al., 1997). The consequences of N binding to embryonic ERβ is unknown, however, N added to uterine explants exhibited a slightly distinct action on AR gene expression than T. This could be related to N acting not only through AR but also through ERβ.

Our data are summarized in Fig. 6-2. Peri-implantation embryos in the uterus begin to secrete estrogens (Gadsby et al., 1980; Pusateri et al., 1990) and possibly 19-nortestosterone (Kao et al., 2000) around 11 days after fertilization. Estrogens stimulate secretion of uterine IGF-I, which then targets the embryo through its IGF-I receptor (Green et al.,1995) to increase the expression of genes associated with cell growth such as cyclin D1. Estrogens in the uterine lumen also act on the embryo primarily through ERβ which leads to a increase in the expression of ERβ as well as P450 aromatase type III genes at the time of embryonic elongation, an event associated with both cell remodeling (Geisert et al., 1982) and cell growth (Ka et al., 2001). The consequence of E binding to embryonic ERβ is to slow cell proliferation, possibly to ensure that mistakes in DNA replication will not occur during accelerated cell division for optimal coordinated maternal and embryo growth. Simultaneously, N secreted by the embryo and androgens in uterine fluids also target the uterine endometrium to modulate those genes stimulated by E, such as IGF-I, thereby regulating uterine

proliferation (Fig. 6-2). The transient reduction in uterine growth could be a requirement for optimal synchronization of embryos and uterus for initiation of implantation.

Collectively, these results provide a basic understanding of the role of estrogens in embryo development and point out a natural selection process where estrogens produced from more advanced embryos impair the development of less developed embryos. Moreover, the action of androgens as mediators of uterine gene regulation as well as growth during early pregnancy provide evidence for the importance of embryo-maternal communication in pregnancy. Our studies could lead to pharmacological strategies for increasing pregnancy success in pigs, as well as provide clues to the etiology of implantation defects during early pregnancy in humans.

For future experiments, the isolation of the regulatory region of ERβ will provide an opportunity to characterize how ERβ is regulated during embryo development and possible factors that modulate its expression. The use of immunoprecipitation experiments to identify proteins that can interact physically with ERβ during embryo development will provide further clues of ERβ functions. It will be important to determine if there are more ERβ isoforms expressed by the embryo. Isolation of new isoforms will allow the design of in vitro experiments to characterize their function using the pTr2 cell line. It will be important to determine the phosphorylation status of the ERβ in different embryo morphology, this could provide further information on regulation of ERβ function Additionally, in vivo experiments could be performed using pregnant pigs treated with

estrogenic compounds which have higher affinity for ERβ than for ERα such as genistein and determine what effect this compound may have on embryo development.

To further study the function of AR in the pregnant endometrium, the use of primary cell culture will allow investigation of the interaction between ERs and AR in transfection experiments using reporter constructs containing estrogen response elements and determine what type of interaction occur among these receptors on the reporter construct activity. Also, utilization of immunoprecipitation to determine which proteins are interacting with AR in endometrial cells will be important. All these experiment will provide a better understanding of these steroid receptors in the regulation of pig pregnancy.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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